Detection and prevention: Improving techniques to manage *Phytophthora agathidicida*, the causal agent of kauri dieback

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Detection and prevention: Improving techniques to manage *Phytophthora agathidicida*, the causal agent of kauri dieback

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Phytophthora agathidicida is the causal agent of kauri dieback, which threatens fragmented, remnant kauri (*Agathis australis*) forests in Aotearoa|New Zealand. Presently, there are persistent knowledge gaps in relation to detection and prevention of kauri dieback, which this study aims to address. Molecular detection methods for *P. agathidicida* using real-time polymerase chain reaction (qPCR) and loop-mediated isothermal amplification (LAMP) have been published, however, they are not yet optimised for testing environmental samples, such as soils. We modified the DNA extraction methods from the qPCR protocol, with a focus on improving both cell lysis and extract purity. We then compared the efficacy of DNA recovery between a manual DNA extraction method and two commercial DNA extraction kits. Soil shaking with a mechanical apparatus that contained ball bearings improved the yield of extracted DNA over handshaking, although there was no significant difference in yield when comparing glass beads to steel ball bearings of different sizes. Despite efforts to reduce the co-precipitation of humic acids with DNA, extract purity remained too compromised for downstream analysis without the use of a commercial clean-up kit. *P. agathidicida* was detected in all manually extracted samples, whereas detection with commercial extraction kits was inconsistent.

The second part of this study addressed dieback prevention by testing the efficacy of four anti-oomycete fungicides along with five essential oils (EOs) on *P. agathidicida* mycelial growth. The sensitivity range and average values (in parentheses) of fungicide concentrations that reduced mycelial growth by 50% (EC_{50}) for ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin were 0.072 to 0.104 µg/ml (0.087), 0.303 to 0.414 µg/ml (0.369), 0.018 to 0.022 µg/ml (0.020), and 1.30×10^{-4} to 1.70×10^{-4} µg/ml (1.55×10^{-4}), respectively. Exotic plant EOs (*Thymus vulgaris* and *Pelargonium graveolens*) more effectively inhibited mycelial growth than indigenous plant EOs (kānuka and mānuka), although essential oils as a whole were significantly less effective at reducing mycelial growth when compared to fungicides. Several compounds identified in these essential oils by gas chromatography-mass spectrometry warrant further screening against *P. agathidicida* due to their known antimicrobial properties. The findings of these chemical trials may offer alternative or supplementary treatments to phosphite injections, which are the only know treatment, but not cure, for kauri dieback. Together, these experiments aim to improve *P. agathidicida* detection and prevention techniques, which are critical components of disease management and essential for the long-term survival of kauri.

Keywords: *Phytophthora agathidicida*, kauri (*Agathis australis*), kauri dieback, disease management, antimicrobial activity testing, soil DNA extraction.

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1.1 Overview of the New Zealand kauri (*Agathis australis*)

1.1.1 Scientific classification

Araucariaceae is an ancient family of coniferous trees (Kershaw & Wagstaff, 2001) comprised of three genera — *Agathis*, *Araucaria*, and *Wollemia* (Lu et al., 2013). Araucariaceae reached peak diversity and global distribution during the Jurassic period (Miller, 1977), but species diversity has declined since the Cretaceous period due to drier and more variable climates (Miller, 1977). Today, Araucariaceae is considered to be a relictual family with only 41 species (Miller, 1977), which are confined to tropical and subtropical regions in South America, Southeast Asia, and Pacific Islands (Kershaw & Wagstaff, 2001), with the genus *Agathis* only present in the south-west Pacific region (Miller, 1977). Kauri (*Agathis australis*) is the sole member of the Araucariaceae family present in Aotearoa|New Zealand, to which it is endemic (Ecroyd, 1982).

1.1.2 Description of kauri

Kauri (*Agathis australis*) is one of the world's largest and longest living trees (Ahmed & Ogden, 1987; Ecroyd, 1982). The average stem of a mature kauri ranges between 12 and 25 m in height, although total height when including the crown can extend up to 60 m (Steward & Beveridge, 2010). The exact longevity of kauri has been difficult to determine, as the oldest kauri tend to have partially hollow stems (Steward & Beveridge, 2010). That being said, it has been estimated that the average lifespan of mature trees is 600 years, with older trees persisting for longer than 1,500 years (Ahmed & Ogden, 1987; Enright & Ogden, 1995).

These trees produce lanceolate, olive-green leaves (Ecroyd, 1982), which are typically functional for between 3 and 10 years (Ogden & Ahmed, 1989; Silvester & Orchard, 1999), but may remain productive for as long as 15 years (Silvester & Orchard, 1999).

Juvenile kauri has a well-developed taproot (Morrison & Lloyd, 1972), which disappears with maturity and is replaced with peg roots, that deeply penetrate into soil to anchor the tree, and fine feeding roots, that are distributed through the litter and raw humus soil layers (Steward & Beveridge, 2010). Mature kauri have an extensive lateral root system, which often extends beyond its own canopy crown (Steward & Beveridge, 2010). In instances where the root systems of two kauri overlap, root grafting, or fusion, can occur (Bader & Leuzinger, 2019; Ecroyd, 1982; Hill et al., 2017; Hillary, 1944), which

allows for the transmission of various materials between trees (Epstein, 1978). This can include the transmission of pathogens (Epstein, 1978), and it has been documented that *Phytophthora* pathogens can spread from tree to tree this way (Commonwealth of Australia, 2017; Epstein, 1978; Gordon, 1974; Scott & Williams, 2014).

Kauri trees are stressed by lower temperatures and wetter, cloudier environments. Conversely, kauri growth is enhanced with drier, sunnier weather, which in Aotearoa typically commences in September or October and ends in April (Ogden et al., 1992).

1.1.3 Keystone status

The term 'keystone species' is used to describe organisms that influence community dynamics, ecosystem processes, and both population growth and distribution (Ellison et al., 2005). The kauri tree species meets this definition due to its influence on ecosystem processes, particularly those related to soil, and their effect on species composition (Wyse et al., 2014).

Kauri has a profound impact on both litter and soil quality. In the forests they inhabit, kauri can produce the majority of litter inputs. Only about 35% of this input is leaf biomass, and instead, kauri predominately shed twigs, branches, and reproductive structures (e.g., cones) (Silvester & Orchard, 1999). The average residence time of kauri litter is 9 to 78 years, which leads to the formation of a deep litter layer in kauri forests (Jongkind et al., 2007). Prior to shedding leaves and branches, kauri resorb nutrients from these tissues (Ogden & Stewart, 1995; Silvester, 2000), leading to low nutrient availability in kauri soils, particularly with regard to calcium, phosphorus, and nitrogen (Jongkind et al., 2007; Molloy, 1998; Ogden & Stewart, 1995; Verkaik & Braakhekke, 2007). Decomposition of kauri litter leads to the release of polyphenols, the most abundant of which are tannins and acidic, humic substances. Tannins reduce nitrogen mineralisation in the soil, which reduces nitrogen availability (Verkaik et al., 2006), while humic acids are attributed with lowering the soil pH and causing soil leaching, soil podsolisation, and gleying processes (Ecroyd, 1982; Jongkind et al., 2007; Molloy, 1998). The fine feeding root systems of kauri are located in both the leaf litter and humus layers (Ecroyd, 1982; Steward & Beveridge, 2010), which efficiently take up available water and consequently leave the organic soil layer under kauri drier than that of tea tree and angiosperm forests (Verkaik et al., 2007; Verkaik & Braakhekke, 2007).

The influence kauri exhibits on litter and soil quality greatly impacts the plant community in kauri forests. The low soil pH under kauri — approximately pH 4 in litter and pH 3 in organic soil — prevents species sensitive to acidic soils from establishing (Wyse & Burns, 2013). There are additional, although unspecified soil characteristics in the litter, organic, and mineral soil layers in kauri forests that inhibit the germination and growth of certain plant species, which suggests that kauri litter or roots produce

phytotoxic compounds that have an allelopathic effect on some plants (Wyse, 2012; Wyse & Burns, 2013). As this phenomenon has been observed across geographically distinct locations, it indicates that the species observed in kauri forests are likely shaped by kauri themselves (Wyse et al., 2014).

Despite the infertile and likely phytotoxic soils conditions that kauri creates, kauri forests are more diverse than any other forest type in Aotearoa (Ogden, 1995; Wardle, 2002). Approximately 90 species — including insects, reptiles, birds, minor flora, and trees — have been identified in relation to kauri forests (Chetham & Shortland, 2013). Many of the plants that are most positively correlated to kauri forests are also endemic to Aotearoa (Ogden & Stewart, 1995) and include rimu (*Dacrydium cupressinum*), rewarewa (*Knightia excelsa*), nīkau (*Rhopalostylis sapida*), and kānuka (*Kunzea ericoides*), among many others (Nicholls, 1976; Wardle, 2002; Wyse et al., 2014).

The influence of kauri on species diversity also extends into the soil. Kauri roots form mycorrhizal relationships, specifically with arbuscular mycorrhizas (Ecroyd, 1982; McKenzie et al., 2002; Steward & Beveridge, 2010). The majority of these arbuscular mycorrhizas belong to the Glomeromycota class, and some are thought to be uniquely associated with kauri (Padamsee et al., 2016).

1.1.4 History and current state

Kauri has always been treasured by Māori for its cultural, ecological, and spiritual value (Ministry of Primary Industries, 2014; Nuttall et al., 2010). Prior to European colonisation, kauri forests covered more than one million hectares and few kauri would have been harvested for timber (Steward & Beveridge, 2010). Those that were would have used for carving of waka|canoe (Steward & Beveridge, 2010). During this time, Māori considered kauri resin to be a more valuable resource than kauri timber, which was used to start or fuel fires as well as the act as a colouring agent for tā moko|traditional Māori tattoos (Steward & Beveridge, 2010).

Conversely, early European colonisers primarily valued kauri for its timber and economic potential (Nuttall et al., 2010). Kauri timber was harvested by Europeans as early as 1722 and its timber was seen as particularly valuable as it was resistant to decay and tolerant of moist conditions (Steward & Beveridge, 2010). Thus, harvested kauri timber was used extensively in construction and was incorporated into the manufacture of boats, bridges, railways, mine props, and carpentry (Ogden & Stewart, 1995; Steward & Beveridge, 2010).

By 1873, the population of kauri had declined by 70% (Steward & Beveridge, 2010), although logging efforts didn't peak until 1905 (Wardle, 2002). During this time, logging efforts primarily focused on ricker and mature kauri trees (Ahmed & Ogden, 1987), however, this did not entirely spare juvenile or extremely large, 'over-mature' trees from harm. While not felled, 'over mature' kauri were damaged

to collect kauri resin, which could lead to infections and secondary rots (Steward & Beveridge, 2010; Wardle, 2002), and juvenile kauri were frequently cleared by burning (Ogden & Stewart, 1995).

In the 1920s, both kauri logging and resin collection had declined, before completely ceasing on Crown land in 1981 (Steward & Beveridge, 2010). Rather than allowing deforested land to naturally regenerate, these areas were typically converted into exotic pine forests (New Zealand Department of Conservation, 2011; Steward & Beveridge, 2010).

The present day natural range of kauri is north of 38°07'S on Te Ika-a-Māui|the North Island as well as offshore islands, including Aotea|Great Barrier Island and Te Hauturu-o-Toi|Little Barrier Island (Ecroyd, 1982) (Figure 1.1). Kauri today is found in three forest types — kauri forests that are either dominated by ricker stands or old-growth forests, kauri-softwoods-hardwoods forests, and kauri-softwoods-hardwoods-beeches forests (Nicholls, 1976). Kauri is most commonly found in Te Tai Tokerau|Northland and Te Tara-o-te-ika a Māui or Te Paeroa-a-Toi |Coromandel Peninsula and Ranges as well as islands to the east of Te Ika-a-Māui (Steward & Beveridge, 2010), where it is generally confined to high-plateaus, spurs, and ridges (Ogden & Stewart, 1995; Wardle, 2002). While it is possible that kauri growth would be supported by a more southern climate in Aotearoa, it appears that soil and terrain conditions restrict its advance (Ogden et al., 1992).

Old-growth kauri forests cover approximately 1 to 5% of their pre-colonisation area (Ministry of Primary Industries, 2014; Ogden & Stewart, 1995), with the largest stand of mature, old-growth kauri located in Te Tai Tokerau (Ecroyd, 1982). These forests are highly fragmented (Ahmed & Ogden, 1987), which has serious implications for their conservation. Forest fragmentation results in an increased perimeter to area ratio, which can lead to greater representation of peripheral or 'edge' communities and consequently altered species composition and growth phases (Young & Mitchell, 1994).

Current kauri management has primarily focused on regenerating forests through the planting of nursery seedlings (Steward & Beveridge, 2010). Over the past 40 years, regeneration has been promoted by councils, landscapers, environmentalists, and home gardeners (Nuttall et al., 2010). Today, regenerating kauri stands cover approximately 60,000 hectares (Ministry of Primary Industries, 2014). While regeneration has been largely successful, there are a few factors that hinder its progress. Insect activity destroys a large proportion of kauri seeds (Mirams, 1957) and herbivory from both birds and mammals damages kauri reproductive structures and destroys seedling populations (Ecroyd, 1982).

The health of remaining, mature kauri is also at risk. Kauri trees are vulnerable to a number of pathogens, which affect root, stem, and leaf biomass (Ecroyd, 1982; McKenzie et al., 2002). These diseases are primarily caused by fungal or fungal-like pathogens, including members from the

Armillaria, *Fusarium*, *Phytophthora*, and *Pythium* genera (Ecroyd, 1982; McKenzie et al., 2002). These diseases can stem from primary infections, the direct penetration of fungal hyphae into healthy tissue, or from secondary infections as a result of superficial bark and root damage due to insects, birds, and mammals herbivory or previous fungal infections (Ecroyd, 1982; Steward & Beveridge, 2010). Until recently, pathogens and pests of kauri were only considered to have low or local impacts on kauri health (Steward & Beveridge, 2010), with *Phytophthora* pathogens causing the most damage (McKenzie et al., 2002).

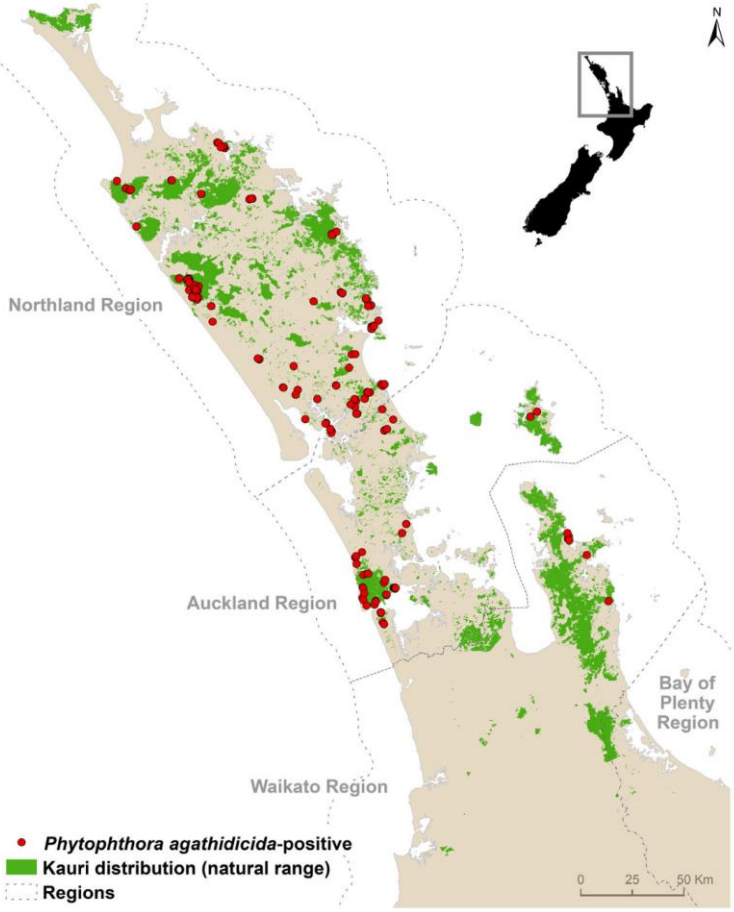

Figure 1.1: Present distribution of kauri (*Agathis australis*) in Aotearoa|New Zealand
The presence of kauri is shown in green overlays while red dots represent locations in which the causal agent of kauri dieback has been detected. Figure reprinted from Bradshaw et al. (2019).

1.2 Overview of the *Phytophthora* genus

Phytophthora, which translates to "plant destroyer" in Greek, are oomycetes that are globally responsible for a variety of plant-damaging diseases (Judelson & Blanco, 2005). While *Phytophthora* are colloquially described as fungi, they are in fact water moulds and more closely related to brown algae (Erwin & Ribeiro, 1996). Consequently, there are a number of key features that distinguish

oomycetes from true fungi, including cell wall composition, septa presence, motility of asexual spores, and the type of sexual spores produced (e.g., oospores, ascospores, etc.) (Erwin & Ribeiro, 1996; Judelson & Blanco, 2005).

The most recent *Phytophthora* genus review described 142 formally named species along with 43 provisionally described species across 10 clades (Blair et al., 2008; Martin et al., 2012; Yang et al., 2017), although additional *Phytophthora* species have since been identified (Mideros et al., 2018) and it is estimated that there may be between 200 to 600 extant species (Brasier, 2009).

This genus has been regularly associated with nursery, forest, and ecosystem damage, with approximately 38% of identified species considered pathogenic (Brasier, 2009). Notable pathogens include *P. cinnamomi* (Jarrah Dieback in the Australian bushland) (Newhook & Podger, 1972), *P. ramorum* (Sudden Oak Death in both North America and Europe) (Grünwald et al., 2012), and *P. pinifolia* ('Daño Foliar del Pino' in Chile) (Durán et al., 2008).

To date, 31 *Phytophthora* species have been identified in Aotearoa, although the origin of most of these species is unknown (Lewis et al., 2019b; Scott & Williams, 2014). *Phytophthora* in Aotearoa affect plants in agricultural, exotic forests, and natural ecosystems and typically spread through either soil-borne spores or root grafting (Scott & Williams, 2014).

1.3 Interaction between kauri and *Phytophthora* pathogens

Phytophthora species have been isolated from kauri stem and root tissue as well as kauri forest soils since the late 1950s. *P. cinnamomi*, *P. cryptogea*, and *P. nicotianae* (= *P. parasitica*) were first reported as pathogens of kauri in 1959 (Brien & Dingley, 1959; Newhook, 1959). Nearly two decades later, *P. agathidicida* was detected in kauri tissue on Aotea (Gadgil, 1974), which was subsequently reported widely on the mainland of Te Ika-a-Māui in 2006 (Beever et al., 2009). More recently, *P. kernoviae* and *P. multivora* have been isolated from kauri forest soil (Ramsfield et al., 2007; Waipara et al., 2013).

Phytophthora pathogens usually cause minor root damage to kauri and, until recently, were not typically associated with tree death (Horner & Hough, 2014a; McKenzie et al., 2002). However, under certain environmental circumstances, such as prolonged rainfall, some *Phytophthora* pathogens, particularly *P. cinnamomi*, can cause tree mortality (Beever et al., 2009; Newhook & Podger, 1972; Scott & Williams, 2014). Furthermore, in instances where multiple *Phytophthora* pathogens are present, it is possible that the pathogens can act synergistically to cause greater harm than they would individually, which could kill a host (Waipara et al., 2013).

For kauri with dieback symptoms, *P. agathidicida* is the most common *Phytophthora* spp. recovered from soil samples, although *P. cinnamomi*, *P. cryptogea*, *P. multivora*, and *P. nicotianae* have also been

detected at much lower frequencies (Bellgard et al., 2013; Waipara et al., 2013). Specifically, the occurrence of *P. multivora* has been associated with a select number of sites that experienced sudden collapse and kauri mortality (Waipara et al., 2013). However, in diseased kauri stem cambium tissue, *P. agathidicida* has been the only *Phytophthora* pathogen recovered (Scott & Williams, 2014; Waipara et al., 2013).

1.3.1 Kauri dieback

Kauri dieback, previously called 'kauri collar rot' (Beever et al., 2009), presents as lesions or cankers that form on the lower tree trunk that spread over time to effectively girdle and kill the tree (Beever et al., 2009).

Infection begins in the root system, which likely reduces the tree's capacity to uptake water and nutrients from the soil. This damage can cause an initial up-regulation in the photosynthetic capacity of leaves to compensate for reduced access to labile soil carbon (D'Souza et al., 2021), however, over time the canopy yellows (Figure 1.2a) and thins (Figure 1.2b & 2c). After a latent period in the root system, the pathogen spreads to stem cambium tissue and causes lesions. These lesions damage the phloem resin canals, which induces the production of resin from the trunk, a process called 'resinosis' (Figure 1.2d) (Beever et al., 2010). Taken together, the damage to the roots, trunk, and canopy eventually causes tree death.

Of these symptoms, the presence of lesions and resinosis are the only diagnostic features that are exclusively associated with kauri dieback. The other symptoms listed above can occur with environmental stress or in the presence of other plant pathogens (Beever et al., 2010). That being said, as kauri respond to mechanical injury and insect activity through resin production, these dieback symptoms may be overlooked or misdiagnosed (Beever et al., 2010). Furthermore, there is reportedly variation in kauri dieback symptoms. Specifically, heavily infected kauri trees located in Glenbervie and Punaruku have been recorded as indistinguishable from healthy trees, which suggests that disease presentation could be influenced by external environmental factors (Beauchamp, 2013).

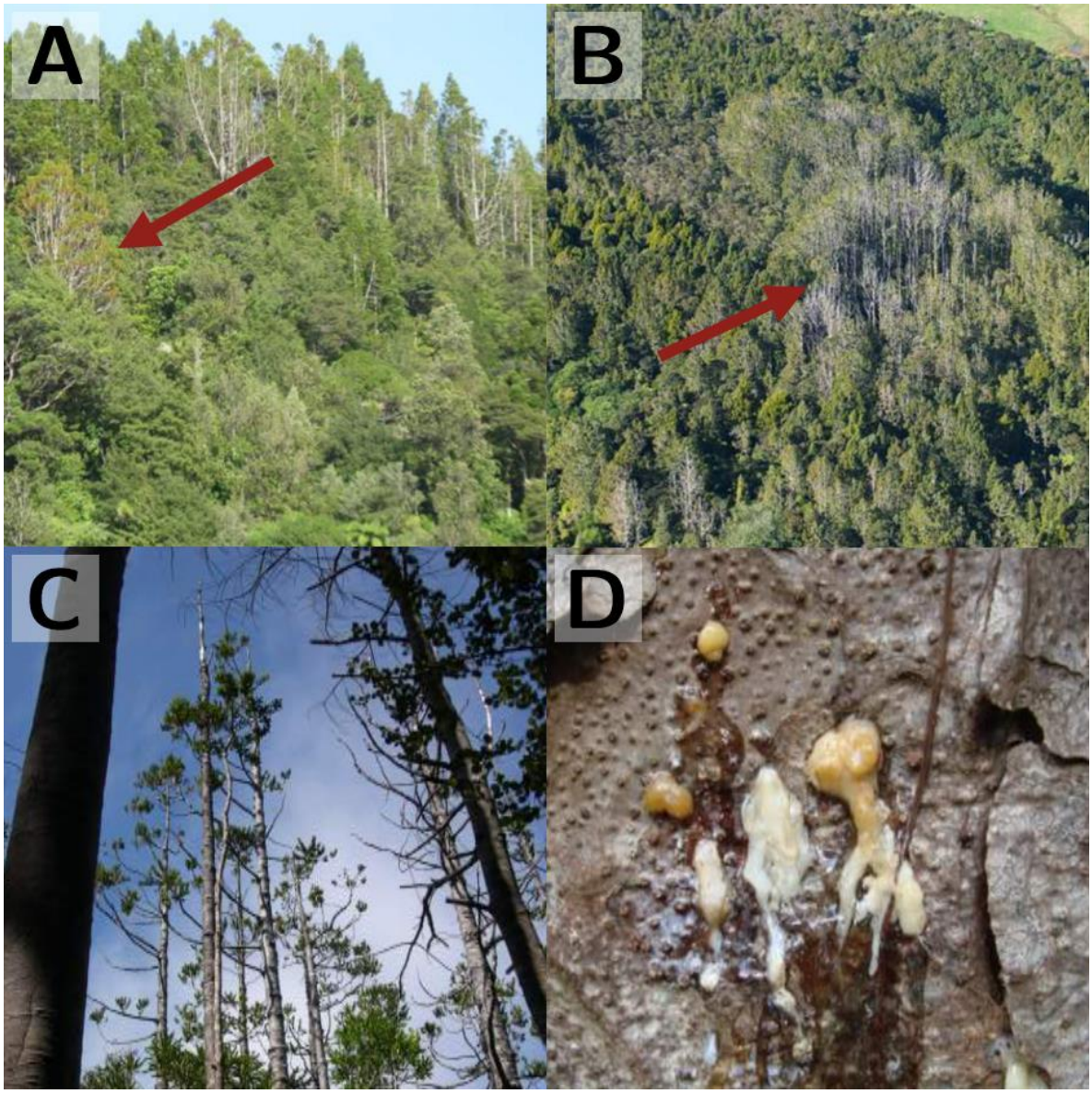

Figure 1.2: Symptoms of *Phytophthora agathidicida* infection, or kauri dieback, in kauri
A| Yellowing of kauri canopy foliage, B| 'ill thrift' trees with a thinning canopy, C| loss of canopy leaves and branchlets, and D| trunk lesion with resinosis. All images are adapted from Beever et al. (2010).

The first documented occurrence of kauri dieback is attributed to a stand of trees in 1972 in the Aotea Conservation Park, which presented with dead seedlings, discoloured foliage, and bleeding cankers (Gadgil, 1974). Just over three decades later, kauri dieback was officially reported on Te Ika-a-Māui, although there had been anecdotal reports of possible dieback spanning from the 1970s to 1990s (Beauchamp, 2013; Waipara et al., 2013). Kauri dieback has now been reported in the Waitakere Ranges, Whangarei, Aotea, Rodney, Pakiri, Albany, Raetea, Trounson, and Punaruku (Bellgard et al., 2013). Thus far, the disease has not been found in the Te Tara-o-te-ika-a-Māui, Hunua Ranges Regional Park, or off-shore islands other than Aotea (Beauchamp, 2013; Bellgard et al., 2013).

1.4 *Phytophthora agathidicida*

During the first reported instance of kauri dieback in 1972, morphology studies identified the causal agent as *P. heveae* (Gadgil, 1974). As early as 2007, scientists distinguished the causal agent of kauri dieback from *P. heveae* through ITS sequencing (Beever et al., 2009). Shortly afterwards, the pathogen was declared an Unwanted Organism in Aotearoa under the Biosecurity Act (1993) (Ministry of Agriculture and Forestry, 2008).

Until 2015, the pathogen was provisionally called *Phytophthora* taxon *Agathis* or 'PTA' (Beever et al., 2009), after which the *Phytophthora* clade 5 was revised. The pathogen was then taxonomically named *P. agathidicida* (Weir et al., 2015), which means '*Agathis*-killing *Phytophthora*' (Bellgard et al., 2013).

The origin of *P. agathidicida* is still debated (Beever et al., 2010; Black & Dickie, 2016; Weir et al., 2015). Aspects of the kauri dieback pathology (e.g., high mortality in kauri (Horner & Hough, 2014a)) and history (e.g., first observed approximately five decades ago) suggest that the pathogen is non-native (Beachman, 2017; Black & Dickie, 2016). If so, it is possible that *P. agathidicida* was introduced to Aotearoa via soil ballast from timber ships, which would place the introduction of the pathogen in the 1900s (Bellgard et al., 2013). The subsequent spread of the pathogen could then be attributed to the movement of contaminated soils with human activities (e.g., tyre treads and shoes) (Bellgard et al., 2013) or animal behaviours (e.g., feral pig foraging) (Krull et al., 2013), or through the movement of infected plant material from nurseries (Beachman, 2017; Beauchamp, 2013; Waipara, 2018).

Alternatively, a recent comparison of mitochondrial genomes between *P. agathidicida* isolates from different regions of Aotearoa with other members of *Phytophthora* clade 5 suggested that the pathogen was introduced at least 300 years ago and has since diversified in Aotearoa (Winkworth et al., 2021). It is worth considering, however, that *Phytophthora* clade 5 currently contains only five species (Weir et al., 2015), making it the most poorly understood *Phytophthora* clade. As genetic analyses may be heavily influenced by as of yet-unknown members of this clade, it is difficult to know the centre of diversity of *Phytophthora* clade 5 or be certain in species age estimates (Weir et al., 2015; Winkworth et al., 2021). If *P. agathidicida* has in fact been in Aotearoa for centuries, it suggests that the relatively recent onset of kauri dieback is likely attributed to environment change (e.g., landscape use changes) and overall decreases in kauri health since colonisation (e.g., environmental stressors and forest fragmentation), which has amplified the severity and spread of the pathogen.

1.4.1 Life cycle of *Phytophthora agathidicida*

Phytophthora agathidicida is a soilborne root pathogen that infects the root systems of many native and exotic plants in Aotearoa, including rimu, mamangi (*Coprosma arborea*), pōhutukawa (*Metrosideros excelsa*), rewarewa, mānuka (*Leptospermum scoparium*), kānuka, porokaiwhiri

(*Hedycarya arborea*), tawa (*Beilschmiedia tawa*), tarairi (Beilschmiedia tarairi), korokio (*Corokia buddleioides*), and kauri (Bellgard et al., 2016; Lewis, 2018; Ryder et al., 2016). The pathogen is present throughout the organic soil layer and in the upper 15 to 20 cm of mineral soil in infected areas, however, there can be significant heterogeneity in horizontal spatial distribution of the pathogen around hosts (Bellgard et al., 2013; Singh et al., 2017). At Aotea, *P. agathidicida* was found to naturally spread at a rate of 0.3 m a year (Bellgard et al., 2009), although it spreads at a faster rate of approximately 0.57 m a year in the Waitakere Ranges (Bellgard et al., 2013).

This pathogen primarily spreads via bi-flagellate zoospores (Figure 1.3a), which are motile and are attracted to host roots via chemotaxis (Armstrong, 2018). After making contact with a susceptible root, zoospores encyst and germinate (Figure 1.3b). The subsequently produced germ tube penetrates the root and produces intracellular hyphae (Figure 1.3c), which are simple with slight swellings (Weir et al., 2015), that colonises plant cells. *P. agathidicida* likely first infects fine root structures and then spreads to secondary and primary roots (Bellgard et al., 2013).

Within root tissue, hyphae can either undergo asexual reproduction to produce sporangia (Figure 1.3e), discussed below, or homothallic, sexual reproduction, wherein an oogonium is fertilised by an antheridium. The oogonium has slightly raised protuberances with a mildly stipulate wall, whereas the antheridium is globose, with knots at the base, and amphigynous (Bellgard et al., 2013; Weir et al., 2015). The product of this joining is a thick-walled oospore (Figure 1.3d). Oospores of *P. agathidicida* nearly fill the oogonium, making it the largest oospore of all currently reported *Phytophthora* clade 5 species (Weir et al., 2015).

Oospores germinate and produce terminal sporangiophores that are long, thin, and branched (Weir et al., 2015). These sporangiophores bear sporangium, which are globose or ovoid-ellipsoid in shape, non-caducous, have semi-papillate to papillate pore development, and typically have an length of 30 to 45 µm and width varying from 18 to 28 µm (Bellgard et al., 2013; Weir et al., 2015). Sporangia either cause infection directly, through germination, or indirectly by undergoing zoosporogenesis to release motile zoospores into the environment (Bradshaw et al., 2019; Judelson & Blanco, 2005; Weir et al., 2015).

After an indeterminate latent period in roots, the pathogen moves into the trunk biomass. Here, *P. agathidicida* is most readily recovered from phellogen, or cork cambium, but can also be found in the non-conducting phloem and secondary xylem, or sapwood (Wheat et al., 2012).

While there have been a few reports that *P. agathidicida* also produces chlamydospores (Bellgard et al., 2016; Singh et al., 2017), thin-walled survival spores, this has not been universally observed (Dick & Kimberley, 2013; Weir et al., 2015), and the most recent *P. agathidicida* review does not include it as part of the pathogen's life cycle (Bradshaw et al., 2019).

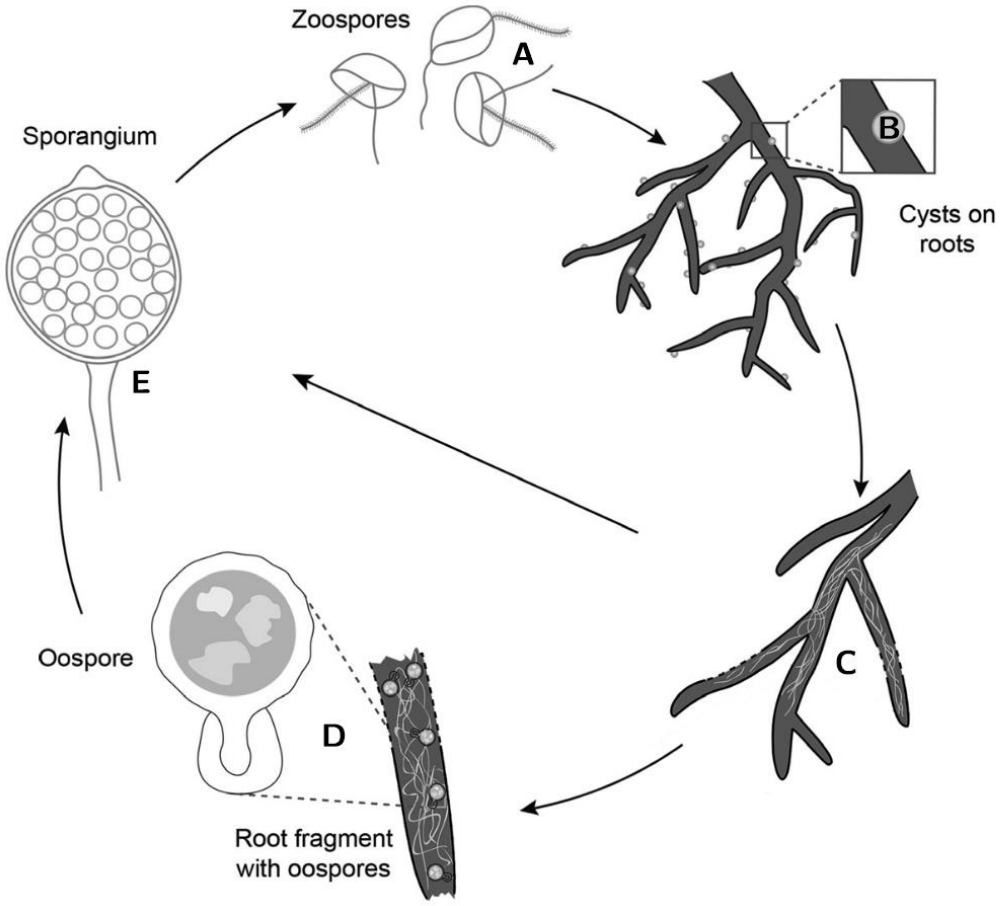

Figure 1.3: Life cycle of *Phytophthora agathidicida*
A| Bi-flagellate zoospores, B| encystment of zoospores on susceptible roots, C| germinated zoospores infect root systems and produce intracellular hyphae. Intracellular hyphae can then produce D| thick-walled oospores or e| sporangia. Adapted from Bradshaw et al. (2019).

1.5 Implications of kauri dieback in Aotearoa|New Zealand

1.5.1 Cultural impacts

Kauri features prominently in Māori creationism beliefs. It is said that at the beginning of the world, Ranginui|The sky father and Papatūānuku|The earth mother were embraced and left the world and all their children in darkness. Tāne, one of their children, grew tall and forced his parents apart, filling the world with light and allowing life to flourish. Many iwi|Māori communities or people consider the legs of Tāne to be kauri (Figure 1.4) (Keep Kauri Standing, 2016; Te Ara - the Encyclopedia of New Zealand, 2007), and prominent kauri today hold names such as Tāne Mahuta|God of the forest and Te Matua Ngahere|Father of the forest (New Zealand Department of Conservation, 2011).

Figure 1.4: A visual interpretation of Tāne separating Ranginui and Papatūānuku
Reprinted with permission from Jane Crisp.

Thus, kauri is considered a taonga|treasured species for many iwi (Nuttall et al., 2010). Some iwi, such as Te Roroa consider themselves kaitiaki|guardians of kauri forests and believe that the wellbeing and health of kauri is a reflection of their own health (Nuttall et al., 2010; Shortland & Wood, 2011). Therefore, kauri dieback has significant cultural implications for Māori mana|spiritual power, tikanga|lore, mauri|life force, hauora|health, and kaitiakitanga|guardianship (Lambert et al., 2018; Nuttall et al., 2010; Shortland & Wood, 2011).

Furthermore, vectors of *P. agathidicida* are poorly quantified, particularly those associated with wildlife and feral pigs. Feral pigs have been suspected of spreading the pathogen, due to their capacity to transport soil on trotters and snouts as well as their rooting behaviour (Bassett et al., 2017; Krull et al., 2013). While this research has not conclusively confirmed feral pigs as a vector, it does have implications for cultural and recreational activities as feral pigs are hunted by some iwi as a source of kai|food (Nuttall et al., 2010; Shortland & Wood, 2011). Similarly, if endemic species, especially those considered to be a taonga, were found to vector the pathogen, it would complicate dieback management strategies (Nuttall et al., 2010).

1.5.2 Ecological impacts

As previously described, kauri is a keystone species that supports the most diverse forest ecosystems in Aotearoa. For kauri infected with *P. agathidicida*, there have already been documented changes in litterfall quality and quantity as well as reproductive capacity (Schwendenmann & Michalzik, 2019; van der Westhuizen et al., 2014). In areas where kauri dieback has spread, there have been noted changes in the composition of regenerative vegetation as well as the community and functional composition of the soil microbial community (Byers et al., 2020; van der Westhuizen et al., 2014).

With regard to ecosystem community changes, it is suspected that widespread kauri death may cause kauri forests to transition into podocarp forests, likely dominated by rimu (Beever et al., 2009). This would likely also affect the abundance and diversity of epiphytes, shrubs, understory trees, reptiles,

insects, and birds in kauri dieback areas (Shortland, 2011; Wyse et al., 2014), although it is not entirely clear to what extent.

Furthermore, kauri provide ecosystem services, such as carbon sequestration, protection from soil erosion, and reduction of flood likelihood (Kauri Dieback Governance Group, 2019). Thus, their loss could accelerate climate change as well as change the physical landscape of indigenous forests.

1.5.3 Financial impacts

Before 2020, Aotearoa received over 300,000 visitors a month (Stats NZ Tatauranga Aotearoa, 2020), which helped employ 8.4% of the population and directly generated 5.8% of total Gross domestic product (GDP) (Stats NZ Tatauranga Aotearoa, 2019). Forests and national parks are considered to be a tourist activity, with 52% of international tourists visiting one during their stay (Ministry of Primary Industries, 2020). In particular, remaining kauri forests are highly frequented, with between 200,000 and 250,000 people per year visiting Tāne Mahuta alone (New Zealand Department of Conservation, 2011; Nuttall et al., 2010), which generates both direct and indirect revenue in the communities they inhabit.

Additionally, as mentioned in Section 1.5.2, kauri provide numerous ecosystem services. These ecosystem services have tangible values, which are expressed in Net Present Value (NPV). For example, carbon sequestration by kauri is valued at 279.3 million NPV (Kauri Dieback Governance Group, 2019). Thus, the loss of these services would not only result in ecological harm but would also have economic consequences.

1.6 Current management strategies for *P. agathidicida*

1.6.1 Public measures to control kauri dieback spread

Traditionally, disease management strategies in forest ecosystems have prioritised vector control, in other words, preventing contact between pathogens and their hosts (Ministry of Primary Industries, 2014). Humans are known vectors for soil-borne pathogenic oomycetes and transport soil on the soles of their shoes (Pau'uvale et al., 2011). To limit the capacity for humans to spread *P. agathidicida*, boardwalks and foot cleaning stations have been installed (Aley & Macdonald, 2018; Lambert et al., 2018). However, despite their implementation, surveys have found that compliance is not yet perfect. There is between 88% and 90% compliance with cleaning stations and 78% compliance with track adherence from visitors in kauri forests (Aley & Macdonald, 2018; Wegner, 2014).

For kauri sites that hold irreplaceable value or for which the cost of boardwalk installations are too high, access to the trees has been completely restricted. For instances in which this arises due to the local and cultural importance of the kauri, the closure is designated as a rāhui|restriction placed by

local Māori (Kauri Dieback Governance Group, 2019; Lambert et al., 2018; Shortland, 2017b). Based on the most current information, at least 31 tracks in Aotearoa are closed for this reason (New Zealand Department of Conservation, 2018).

1.6.2 Mātauranga Māori

Mātauranga Māori refers to indigenous knowledge brought to Aotearoa by either Polynesian ancestors or present-day Māori (Lambert et al., 2018; Shortland & Wood, 2011). In relation to kauri dieback, mātauranga Māori has been considered with regards to determining the overall health of kauri forests, improving kauri dieback detection and monitoring, and identifying indigenous plants and materials that may treat kauri dieback.

The environmental resiliency of kauri toward *P. agathidicida* has likely been lowered due to the extensive kauri harvesting with European colonisation as well as the significant land use conversions from native forests to exotic pine and pastures (Nuttall et al., 2010). Therefore, some iwi have argued that enhancing the overall health of kauri forests (e.g., reducing environmental stressors) is a critical part of kauri dieback management (Nuttall et al., 2010). One way to monitor the health of kauri forests would be through the use of cultural health indicators, which can refer to species abundance or presence, migration patterns, life cycle stages and timings, and larger ecosystem processes that have been identified by Māori as part of a healthy environment (Shortland, 2011). To date, there have been approximately 90 species identified as potential kauri health indicators (Shortland, 2011), and monitoring methodologies are currently being developed and implemented (Chetham & Shortland, 2013; Shortland, 2017a).

Mātauranga Māori has also been applied in natural product screenings from indigenous plants. A wide variety of indigenous Aotearoa plants contain bioactive compounds that display activity against microbes (Bloor, 1995; Calder et al., 1986), perhaps most notably, kānuka and mānuka (Costa et al., 2010; Lis-Balchin et al., 2000; van Klink et al., 2005). For *P. agathidicida* research, there has been a focus on utilising mātauranga Māori to identify pioneer plants that both 'cleanse' and 'prepare' the soil for future generations of plants. While this is still an evolving area of research, soil and plant extracts from mingimingi (*Leucopogon fasciculatus*), kānuka, and mānuka have been shown to inhibit certain life stages of *P. agathidicida* (Kentjens, 2019; Lawrence et al., 2019). Specifically, three flavanone compounds have been isolated from kānuka leaves that inhibit both zoospore motility and germination as well as mycelial growth of *P. agathidicida* (Lawrence et al., 2019).

Rongoā|traditional Māori medicine practitioners have also used mātauranga Māori on diseased trees directly. For example, kauri displaying resinosis in Te Tai Tokerau have been treated with an ointment

containing ground whale bone and spermaceti, which has reportedly reduced resin production and improved bark growth (Ngatae, 2020).

1.6.3 Host resistance

Glasshouse growth trials of kauri infected with *P. agathidicida* have shown that kauri displays very little resistance to the pathogen (Horner & Hough, 2014a). That being said, there are multiple reports of variability in kauri dieback symptoms, both in trees and tissue samples (Beauchamp, 2013; Herewini et al., 2018). This indicates that there may be individuals and populations that have higher resistance to *P. agathidicida*. If such a population was identified, clonal propagation methods have been developed (Gough et al., 2012), which could allow for the amplification of kauri with resistant genotypes.

1.6.4 Chemical disease control

Phosphite (phosphorus acid) and metalaxyl have been traditionally used to control *Phytophthora* pathogens (Gisi & Sierotzki, 2015; Pegg et al., 2002). While metalaxyl only provides slight disease protection to kauri (Horner & Hough, 2013), phosphite injections into kauri trunk biomass has been shown to significantly decrease dieback symptoms, particularly with respect to trunk lesion activity and spread (Horner et al., 2015; Horner & Hough, 2013, 2014b). Despite its relative success, phosphite application has been met with scepticism from some iwi. Te Roroa has expressed concern over the effect that run-off phosphite could have on the larger ecosystem. Specifically, it is not currently know how the presence of phosphite could affect plants, fungi, and microbes associated with kauri or its impact on the availability of kai and the efficacy of rongoā (Nuttall et al., 2010).

In addition to direct treatment, chemical control methods have also been applied to managing disease spread. Disinfectant wash stations have been installed on boardwalks through kauri forests, with the aim of reducing disease spread by human vectors (Bellgard et al., 2009). These stations dispense TriGene II Advance, which effectively inhibits *P. agathidicida* mycelium and zoospores but does not affect oospore viability (Bellgard et al., 2010; Dick & Kimberley, 2013; Pau'uvale et al., 2011). In fact, viable oospores from multiple *Pythium* and *Phytophthora* species have been recovered from the grates of boot wash stations (Pau'uvale et al., 2011). Alarmingly, *Phytophthora* pathogens recovered from these grates were highly persistent and remained viable for at least a year (Pau'uvale et al., 2011).

1.7 Research aims

Despite over a decade of research, there are persistent knowledge gaps in relation to both kauri dieback detection and prevention, arguably two of the most significant aspects of disease control. Therefore, this research project aims to:

1. Optimise the protocol for extracting and amplifying *P. agathidicida* DNA directly from soils as well as determine the detection threshold for different detection methods.

2. Test the effect of four relatively new anti-oomycete fungicides along with five plant essential oil extracts on the growth and viability of *P. agathidicida*.

3. As both research objectives listed above will require the use of *P. agathidicida* oospores, as either a source of inoculum for detection experiments or as a life cycle stage to screen anti-oomycete compounds against in the prevention experiments, this study will also compare different methods for oospore isolation and viability assessments.

Chapter 2:

Phytophthora agathidicida oospore isolation and viability assessments

2.1 Introduction

Oospores are thick-walled, environmentally persistent, resting spores, which are the result of sexual reproduction in oomycetes (Judelson & Blanco, 2005). Oospores are produced through the fusion of male and female gametangia, which are called antheridia and oogonia respectively (Judelson & Blanco, 2005). The position of an antheridium can either be amphigynous, in which the oogonium has grown through the antheridium, or paragynous, where the antheridium attaches to the side of the oogonium (Cooke et al., 2000). Additionally, *Phytophthora* can be classified as either heterothallic, meaning that their gametangia originate from individuals of different mating types, or homothallic, meaning that they are self-fertile (Judelson & Blanco, 2005). *P. agathidicida*, along with approximately half of the species in the *Phytophthora* genus (Judelson & Blanco, 2005), is homothallic (Bellgard et al., 2013). Within a fertilised oogonium, an oospore develops, which contains a well-defined ooplast and may contain multiple pellucid bodies, or nuclei (Figure 2.1).

In the presence of a host or under certain environmental conditions, mature oospores germinate and produce mycelia (Figure 2.2). This process starts when the pellucid bodies within an activated oospore fuse (Erwin & Ribeiro, 1996). Then, the glucans that make up the thick inner wall of the oospore are digested, allowing the protoplasm to fill the spore and eventually move into the newly produced mycelium (Erwin & Ribeiro, 1996).

The presence of oospores in an environmental setting creates a significant challenge for disease management. This structure is long-lived, generally persisting for at least a year, and can survive adverse conditions, such as frost (Erwin & Ribeiro, 1996; Fernández-Pavía et al., 2004). For *P. agathidicida*, oospores have been found to be viable in soil collected from boot wash stations for longer than a year (Pau'uvale et al., 2011) and oospores stored in the laboratory in moist conditions at 10°C were viable for up to nine years (Horner & Hough, 2015).

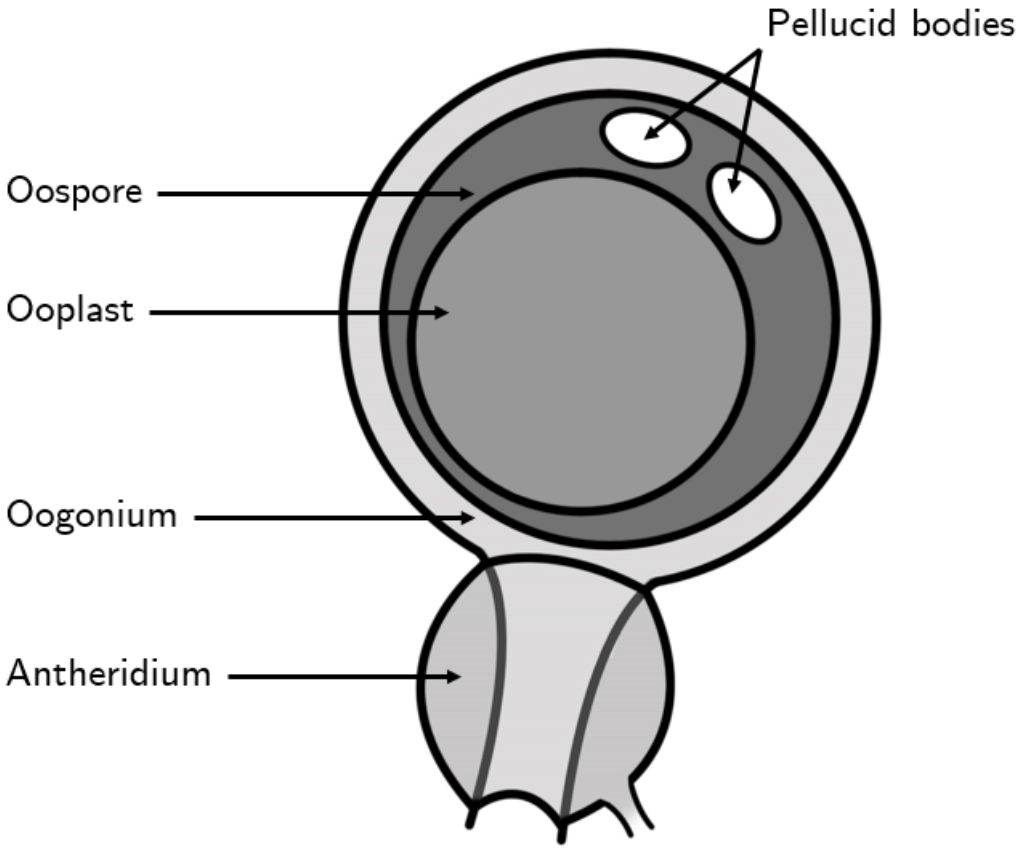

Figure 2.1: Diagram of a mature oospore from the *Phytophthora* genus with an amphigynous antheridium
Key components of the oospore are labelled. Produced by Thurston, (2021).

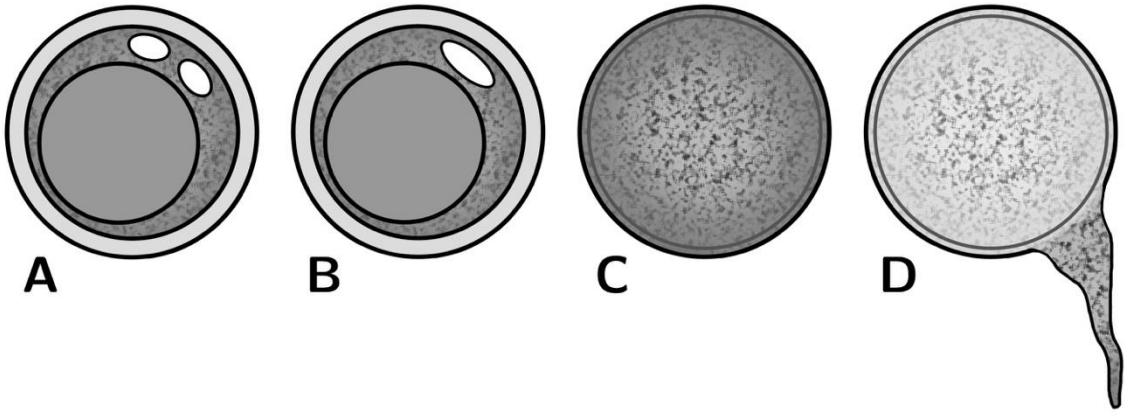

Figure 2.2: Germination process of a *Phytophthora* oospore
A| A mature oospore, with visible ooplast and two pellucid bodies. B| Pellucid bodies fuse. C| The thick inner wall of the oospore has eroded, and protoplasm fills the cell. D| Protoplasm flows into the newly produced mycelium, eventually leaving the original oospore empty. Diagram based on imagery provided by Erwin & Ribeiro (1996) and produced by Thurston (2021).

2.1.1 Oospore production in a laboratory setting

Phytophthora agathidicida oospores have been successfully generated in a number of studies (Bellgard et al., 2013; Dick & Kimberley, 2013; Williams, 2015). Typically, oospores are produced by growing *P. agathidicida* isolates on growth media, either 10% clarified V8 agar (Bellgard et al., 2013) or sterile V8 broth (Bellgard et al., 2009; Dick & Kimberley, 2013; Williams, 2015), in the dark for a prolonged period of time, between 10 days (Bellgard et al., 2013) and eight weeks (Williams, 2015). If *P. agathidicida* oospores are produced in liquid media, they can be further isolated from mycelia through the use of a homogeniser (Bellgard et al., 2009; Dick & Kimberley, 2013; Williams, 2015), although the effectiveness of this has been called into question as the oospore suspension may be contaminated with mycelial fragments (Williams, 2015) (Figure 2.3).

Figure 2.3: Presence of mycelial fragments in a *Phytophora agathidicida* oospore germination study
Arrows indicate oospores that were determined to have produced fresh mycelia. Reprinted from Williams, (2015).

The present inability to completely isolate oospores has two primary consequences. First, it reduces the reliability of germination studies, as it is not entirely clear whether a given oospore has produced new mycelium or if it is simply overlaid on a mycelial fragment. Second, mycelial fragments can affect the calculation of detection thresholds in DNA recovery studies, which rely on a known number of spores, and thus DNA copies, for the inoculum.

2.1.2 Methods for assessing oospore viability

The viability of *Phytophthora* oospores can be determined through exposing oospores to tetrazolium bromide (MTT) dye, inducing oospores to experience plasmolysis, observing morphology, or allowing the oospores to germinate directly on media (Jiang & Erwin, 1990; Sutherland & Cohen, 1983). The

following sections will provide further detail into each of these methods, as well as highlight the difficulties associated with each.

2.1.2.1 Tetrazolium bromide (MTT)

Thiazolyl tetrazolium bromide (MTT) can be used as a vital stain as part of a colourimetric assay. Viable cells reduce tetrazolium salts via mitochondrial dehydrogenases to formazan products, which are strongly pigmented (Cohen, 1984; Stockert et al., 2018). In theory, this assay allows for the differentiation of active (i.e., pre-germination (Jiang & Erwin, 1990) or germinated (Erwin & Ribeiro, 1996)), dormant, and nonviable spores based on the hue that the spores are stained (Table 2.1) (Etxeberria et al., 2011; Jiang & Erwin, 1990).

There are, however, issues associated with colour consistency in MTT assays, arising from either assessor subjectivity or differences in assay chemistry. Assessor subjectivity may influence the terminology used to describe the reaction (Table 2.1, Figure 2.4) (Etxeberria et al., 2011; Williams, 2015), which may be alleviated by assessing MTT assays with a spectrophotometer (Kamiloglu et al., 2020; Riss et al., 2013; Stockert et al., 2018; Williams, 2015). The actual chemistry of the assay can also influence the resulting stain of spores. Specifically, spore pigmentation may be affected by the concentration of chelating ions (e.g., cobalt) (Meier & Charvat, 1993), type of buffer used (e.g., phosphate) (Jiang & Erwin, 1990), concentration of stain applied (Sutherland & Cohen, 1983), and duration of stain application (Etxeberria et al., 2011). Based on the literature, the most apparent impact that this has on viability assessments is the categorisation and presence of blue stained spores (Table 2.1) and the prevalence of black stained spores (Etxeberria et al., 2011).

This assay has high rates of false positives, which is determined by exposing oospores to lethal heat. Across different studies, the false positive rates were as high as 11% (Singh et al., 2004), 15% (Medina & Platt, 1999), 35% (Etxeberria et al., 2011), and 49.6% (Pittis & Shattock, 1994). Furthermore, this assay reportedly has more variation when compared to other methods, namely plasmolysis (Flier et al., 2001), and may not always be directly correlated to oospore germinability (Sutherland & Cohen, 1983).

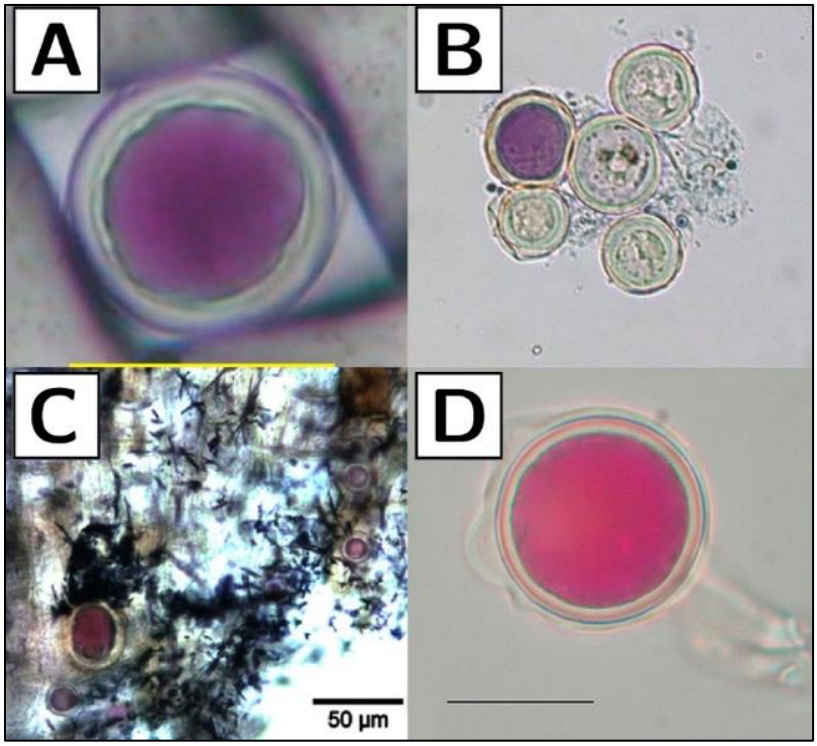

Figure 2.4: Variation in colour and terminology used to describe viable *Phytophthora* oospores identified by the thiazolyl tetrazolium bromide (MTT) colorimetric assay
A| Dormant *P. capsici* oospore stained red to rose. B| Active *P. agathidicida* oospore stained red to purple. C| Viable *P. cinnamomi* oospore, stained magenta. D| Dormant *P. ranorum* oospore, stained purple. Photographs and descriptions reprinted from Etxeberria et al. (2011); Dick & Kimberly (2013); Jayasekera et al. (2007); and Xavier et al. (2010), respectively.

Table 2.1: Colour assessment of *Phytophthora* oospores and ultimate determination of spore viability using the thiazolyl tetrazolium bromide (MTT) colorimetric assay across 16 studies
The description of spore colour was reprinted directly from each study. Hatched cells indicate that there was no information available from a study for the specific viability assessment.

	Viability assessment			
Reference	Active	Dormant	Viable	Nonviable
Sutherland & Cohen, 1983	Blue	Rose		Black / Clear
Beakes et al., 1986	Blue	Rose		Black / Clear
Pittis & Shattock, 1994			Blue / Red	
Medina & Platt, 1999	Blue	Rose		Black / Unstained
Jiang & Erwin, 1990	Blue	Rose		Black / Unstained
Delcán & Brasier, 2001			Pink	Black / Unstained / Blue
Singh et al., 2004			Blue / Red	Unstained
Porter et al., 2007			Red	
Jayasekera et al., 2007			Magenta pink	Black
McCarren et al., 2009			Magenta	Black / Clear
Bellgard et al., 2010	Red	Pink		Black
Xavier et al., 2010)		Purple		Black / Colourless / Blue
Etxeberria et al., 2011	Blue	Red to rose		Black / Unstained
Dick & Kimberley, 2013	Red / Purple	Pink		Black / Unstained / Blue
Hood et al., 2014			Pink / Purple	
Williams, 2015			Pink / Red	

2.1.2.2 Plasmolysis

Plasmolysis is a cellular process in which the nucleus of viable cells retracts as a response to exposure to a hypertonic solution (Figure 2.5). For *Phytophthora* species, plasmolysis has been induced using a 3M sucrose solution (Jiang & Erwin, 1990; Singh et al., 2004) as well as different concentrations of sodium chloride (NaCl) — 2M (Flier et al., 2001; Pittis & Shattock, 1994), 3M (Jiang & Erwin, 1990), and 4M (Etxeberria et al., 2011; Jiang & Erwin, 1990; Kunjeti et al., 2016; Medina & Platt, 1999; Porter et al., 2007; Singh et al., 2004) — although it has been reported that plasmolysis occurs at lower rates when using NaCl concentrations less than 4M (Etxeberria et al., 2011; Jiang & Erwin, 1990). When using NaCl solutions, plasmolysis is rapidly induced (Etxeberria et al., 2011; Medina & Platt, 1999), whereas sucrose solutions require a three day incubation (Jiang & Erwin, 1990). In some studies, despite the use of a hypertonic solution, no plasmolysis was observed (Porter et al., 2007).

While plasmolysis has false positive rates approaching 0% (Etxeberria et al., 2011), it is thought that there is a much higher rate of false negatives (Medina & Platt, 1999), which is harder to quantify. One possibility is that the thick walls of dormant oospores may prevent them from undergoing plasmolysis (Etxeberria et al., 2011; Pittis & Shattock, 1994).

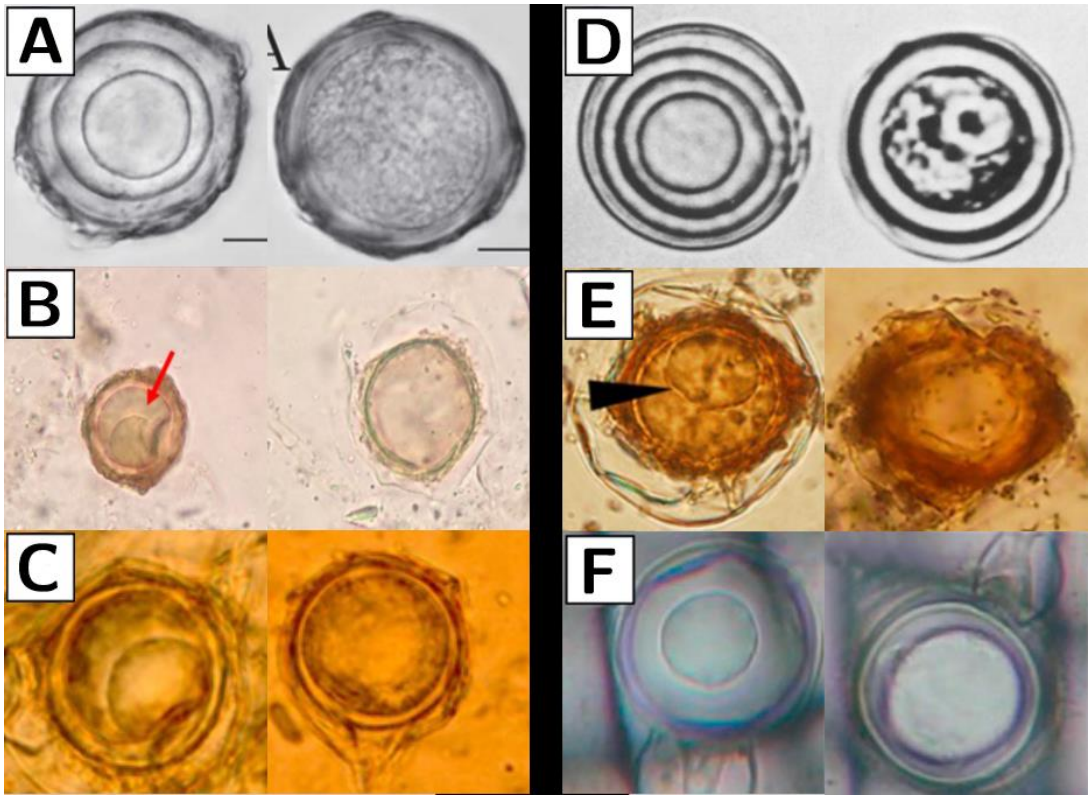

Figure 2.5: Differences between plasmolysed and non-plasmolysed oospores
Each pair of images contains a plasmolysed oospore with retracted nucleus on the left-hand side and a non-plasmolysed oospore on the right-hand side. The species in each set of images are as follows, A| *Peronospora destructor*, B| *Peronospora farinose*, C| *Pseudoperonospora cubensis*, D| *Phytophthora* sp., E| *Peronospora effuse*, and F| *Phytophthora capsici*. Photographs were reprinted from Kusaba & Kawamura (2019); Liu et al. (2018); Thomas et al. (2017); Jiang & Erwin (1990); Kunjeti et al. (2016); and Etxeberria et al. (2011), respectively.

2.1.2.3 Morphological assessments

Even under ideal conditions, *Phytophthora* oospores may be produced that are not viable. While these spores may appear superficially normal, they possess certain attributes that can be used to identify them. Specifically, nonviable oospores tend to have a thicker inner wall and lack an ooplast and nuclei (Figure 2.6) (Erwin & Ribeiro, 1996).

While this observation has been made, it does not appear that any studies use morphological differences between nonviable and viable oospores as a definite measurement of viability.

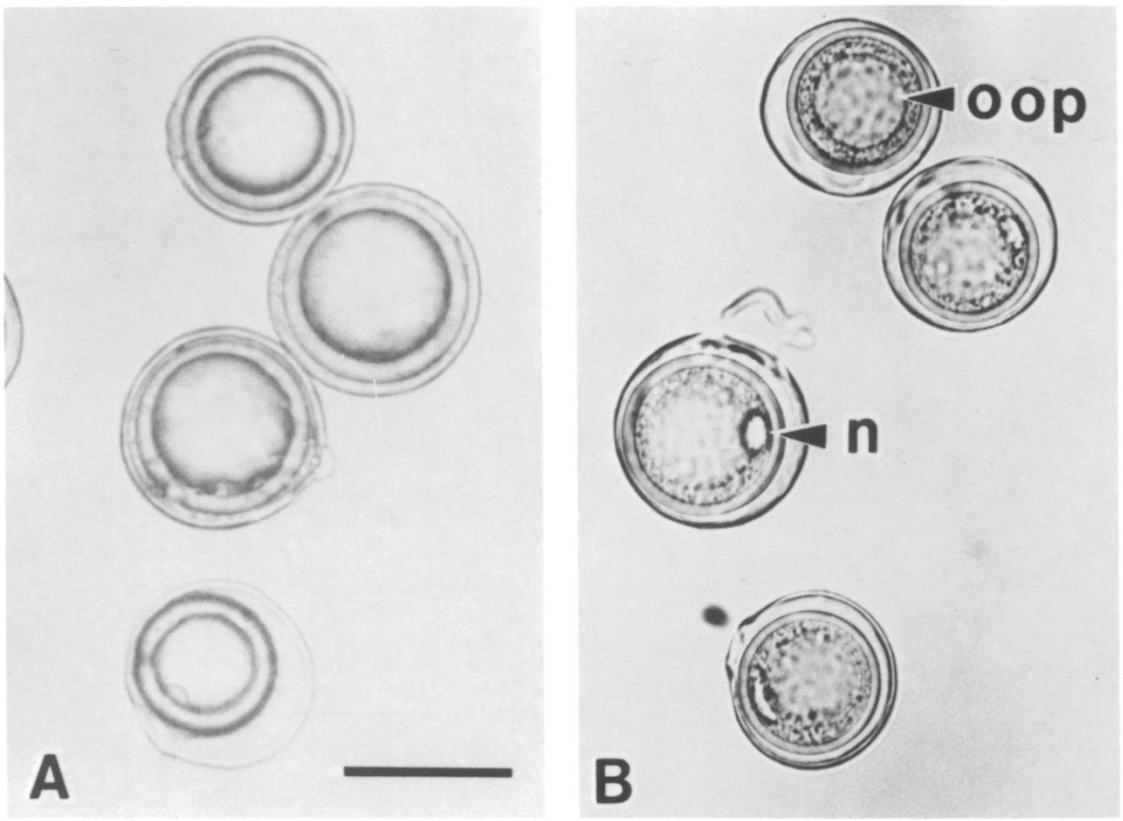

Figure 2.6: Comparison of morphology between nonviable and viable oospores of *Phytophthora sojae*
A| Nonviable and B| viable oospores, showing an ooplast (oop) and nucleus (n). Reprinted from Jiang & Erwin (1990).

2.1.2.4 Germination

Oospores germinate to produce fresh mycelia under various environmental conditions, including host presence, nutrient availability, or environmental chemical signals. Theoretically, direct germination should be the most accurate way to determine oospore viability, although it has been noted that germination may not distinguish between dormant and nonviable spores (Erwin & Ribeiro, 1996). Furthermore, *in vitro* oospore germination may fail in spite of condition optimisation efforts (Jayasekera et al., 2007; McCarren et al., 2009), is highly dependent on species (Delcán & Brasier, 2001), and occurs at particularly low rates for heterothallic species (Xavier et al., 2010). The optimisation parameters adjusted most frequently in oospore germination studies involve the

incubation media used, if any, the photoperiod, and the incubation duration. Some studies report not using any incubation media (Hood et al., 2014; Pittis & Shattock, 1994; Williams, 2015) while others incubated oospore suspensions with soil extracts (Jiang & Erwin, 1990; Medina & Platt, 1999; Sutherland & Cohen, 1983) or on agar media (Delcán & Brasier, 2001; Xavier et al., 2010). For the photoperiod, studies report oospore germination under light with either a 16- (Medina & Platt, 1999) or 24-hour photoperiod (Jiang & Erwin, 1990), as well as in darkness (Delcán & Brasier, 2001). Finally, incubation length ranges from less than a week (Jiang & Erwin, 1990) to two weeks (Hood et al., 2014; Medina & Platt, 1999; Pittis & Shattock, 1994; Sutherland & Cohen, 1983; Williams, 2015) to a month or more (Delcán & Brasier, 2001; Xavier et al., 2010). It has been shown that oospores from different *Phytophthora* species mature and germinate at different rates (Xavier et al., 2010), which likely contributes to these apparent differences in methodologies.

In addition to issues with optimisation, germination studies may be hindered by the presence of mycelial fragments, as described in Section 2.1.1. In one study, this affected as many as 30% of germination assessments (Williams, 2015).

2.1.3 Methods used to assess *P. agathidicida* oospore viability

To date, both germination studies and MTT assays have been used to assess *P. agathidicida* viability, the latter of which has been predominately used. These assessments have been applied to study how *P. agathidicida* oospores are effected by exposure to disinfectants and fumigants (Bellgard et al., 2009, 2010a; Dick & Kimberley, 2013), salinity solutions (Dick & Kimberley, 2013), a range of pH conditions (Dick & Kimberley, 2013), and heat (Dick & Kimberley, 2013; Williams, 2015). Of these four studies, only one utilised germination studies in conjuncture with vital staining, although this assessment was significantly hindered by the presence of mycelial fragments (Williams, 2015) and thus could not describe the correlation between the two.

2.1.4 Proposed study

This study sought to compare the efficacy of methods for isolating oospores from mycelia — including homogenisation, sonication, and enzyme digestion — as well as the accuracy of oospore viability assays, specifically, direct germination, plasmolysis, and MTT staining.

The results of this study have the potential to inform subsequent *P. agathidicida* experiments, as well as general Oomycota research. Furthermore, the methods developed in this chapter will be subsequently referenced in both Chapter 3 and 4.

2.2 Methods

2.2.1 *Phytophthora* isolates

Three *P. agathidicida* isolates were used in this study — NZFS 3770, ICMP 18969, and ICMP 18970. NZFS 3770 was isolated from Te Tara-o-te-ika a Māui in 2006 (Studholme et al., 2016) whereas both ICMP strains were isolated from Waipoua, Te Tai Tokerau in 2011 (Manaaki Whenua Landcare Research, 2013).

2.2.2 Growth media

2.2.2.1 V8 broth

V8 broth was prepared by buffering V8 (V8® Original, Campbell's) with calcium carbonate ($CaCO_3$) (AJA125-500G, Ajax Finechem) at a rate of 1.0 g $CaCO_3$ per 100 mL V8 juice. The buffered V8 juice was further clarified by centrifuging at 3,220 x ***g*** for 20 minutes. The supernatant was decanted and then diluted with reverse osmosis (RO) water to make a 20% (v/v) broth. V8 broth was sterilised prior to use by autoclaving for 20 minutes at 121°C and 15 psi (Jeffers, 2006).

2.2.2.2 Carrot broth

To prepare 1 L of 10% carrot broth, 100 g defrosted frozen carrots were blended with 400 mL RO water in a series of four, 30 second intervals. The blended carrot mixture was strained through two layers of cheesecloth, after which RO water was added to bring the final volume up to 1 L. The carrot broth was sterilised prior to use by autoclaving for 20 minutes at 121°C and 15 psi.

2.2.2.3 Clarified V8 agar

To prepare clarified V8 agar (cV8), 1.5% (w/v) micro agar (M1002, Duchefa Biochemie) was added to 20% clarified V8. The agar was sterilised by autoclaving for 20 minutes at 121°C and 15 psi. Molten media was poured into sterile Petri dishes using aseptic techniques. Plates were set under UV light to further reduce the likelihood of media contamination. After the media had set, the Petri dishes were sealed with Parafilm and stored in the dark at 4°C for up to 6 weeks.

2.2.2.4 *Phytophthora* selective media

Phytophthora agathidicida was cultured on a modified *Phytophthora* and *Pythium* selective growth media. The standard *Phytophthora* selective growth media (PARPH) uses corn meal agar amended with pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB), and hymexazol (Jeffers & Martin, 1986). The modifications implemented in this research project relate to the use of PCNB as an antimicrobial amendment and corn meal agar as the basal nutrient.

PCNB contains trace amounts of the carcinogen hexachlorobenzene (National Toxicology Program, 2016), which has limited its commercial availability. Nystatin, a polyene antibiotic that inhibits the growth of mycopathogens (Hamilton-Miller, 1973), has been found to be a suitable replacement for PCNB (Morita & Tojo, 2007) and has been used with success in previous *P. agathidicida* research (Lewis, 2018). Thus, this study replaced the prescribed 100 mg/L PCNB with 50 mg/L nystatin (Morita & Tojo, 2007). This study further adapted the standard PARPH media recipe by substituting 20% V8 agar as the basal medium, which has been shown to enhance *Phytophthora* spore production (Ferguson & Jeffers, 1999).

The basal medium was composed of 20% clarified V8 concentrate and 1.5% (w/v) micro agar, which was autoclaved for 20 minutes at 121°C and 15 psi. The autoclaved media was cooled in a 55°C water bath, after which it was amended with 200 mg/L ampicillin sodium (A0104, Duchefa Biochemie) dissolved in RO, 50 mg/L nystatin (N0138, Duchefa Biochemie) dissolved in 90% ethanol, 10 mg/L rifampicin (R0146, Duchefa Biochemie) dissolved in acetone, 400 µL 2.5% pimaricin preparation (P0440, Sigma-Aldrich), and 50 mg/L hymexazol (H1348, Tokyo Chemical Industry) dissolved in RO (Jeffers & Martin, 1986).

The molten selective media was poured into sterile Petri dishes using aseptic techniques, which were then set under UV light. After the media had set, the Petri dishes were sealed with Parafilm and stored in the dark at 4°C for up to 6 weeks.

2.2.3 Routine culturing

Phytophthora agathidicida was regularly cultured on 20% cV8 agar. To do so, a 5 mm flame-sterilised cork borer was used to remove an agar plug from the leading edge of a *P. agathidicida* colony. This agar plug was placed in the middle of a Petri dish containing 20% cV8, which was then incubated in the dark at 22°C for 5 to 7 days. This culturing process was repeated eight times before the pathogen was passaged through pear to maintain its pathogenicity.

2.2.4 Maintaining pathogenicity and virulence

Phytophthora isolates maintained in culture collections can lose pathogenicity and virulence over time due to frequent and prolonged culturing (Drenth & Sendall, 2001; Erwin & Ribeiro, 1996). This effect may be more prominent in certain life cycle stages than others, for example, *P. agathidicida* has been found to have reduced zoospore capacity after approximately eight growth cycles on 20% cV8 (Armstrong, 2018).

In order to preserve the pathogenicity of *Phytophthora* cultures, the pathogen should be passaged through a host plant (Drenth & Sendall, 2001), sometimes repeatedly (Sobkowiak et al., 2012). A

previous assessment found that pear tissue more effectively restores *P. agathidicida* pathogenicity when compared to native Aotearoa plant hosts, which should be conducted every six to eight growth cycles (Armstrong, 2018).

To prepare the pear, a rectangular incision was made using a flame sterilised scalpel to effectively produce a mobile flap of tissue (Figure 2.7a). The underlying tissue was then exposed and a 5 mm agar plug containing active *P. agathidicida* mycelia was inserted. The site of insertion was sealed with Parafilm to reduce the occurrence of external contaminants and co-colonisation of the pear. The pear was incubated for approximately five days at 22°C, after which point the tissue surrounding the site of insertion was soft and discoloured (Figure 2.7b). An approximately 1 cm section from the leading edge of the infection was removed, transferred to selective media, and incubated in the dark at 22°C for approximately five days (Jeffers & Martin, 1986). After *P. agathidicida* had been successfully re-isolated on selective media, an agar plug containing active mycelia was transferred onto 20% cV8 and the routine culturing cycle resumed.

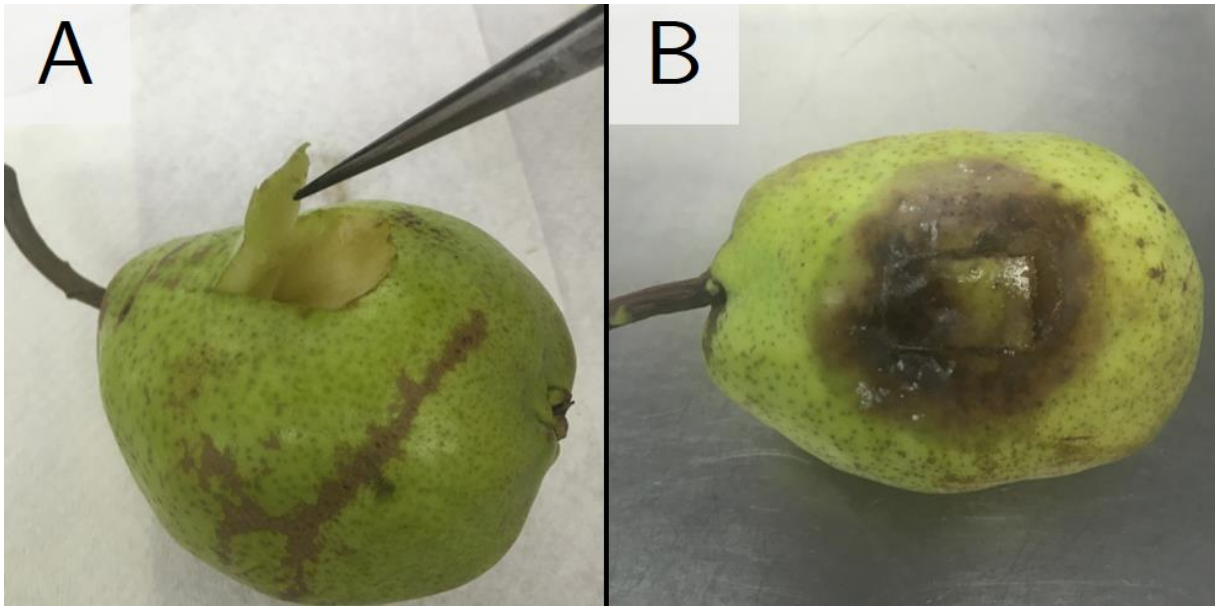

Figure 2.7: Passaging *Phytophthora agathidicida* through pear to maintain pathogen virulence
A| An incision was made in the pear, into which a mycelial plug was placed. B| After approximately five days, the tissue surrounding the incision was discoloured and soft and could be cultured on selective media.

2.2.5 Long-term storage

Prior to passaging *P. agathidicida* cultures through pear, long-term culture stocks were set up for each isolate. This ensured that each isolate could be re-established if the passaging was unsuccessful or if there were contaminants present once the pathogen was cultured from pear tissue.

2.2.5.1 Room temperature in sterile MQ^+

Long-term stocks were set up by placing a 7 mm agar core containing *P. agathidicida* mycelia grown on selective media in a sterile 2 mL polypropylene tube containing 1 mL autoclaved MQ^+ water. These stocks were stored in the dark at room temperature. Long-term stocks were established for *P. agathidicida* isolates NZFS 3770, ICMP 18969, and ICMP 18970.

2.2.5.2 Freezer stocks in glycerol

Freezer stocks were also established by using sterile 50% glycerol rather than autoclaved MQ^+ water and storing the tubes at -80°C. Freezer stocks were established for *P. agathidicida* isolates NZFS 3770, ICMP 18969, and ICMP 18970.

2.2.6 Oospore production

Phytophthora agathidicida oospores were generated by transferring a 5 mm agar plug of active mycelia to the centre of a sterile, 90 mm diameter Petri dish containing approximately 8 mL 20% V8 broth. The plugs should be surrounded, but not covered, by the broth. The plates were sealed with Parafilm and incubated in the dark for between 6 and 8 weeks (Dick & Kimberley, 2013), after which point the Petri dishes were covered in a dense, mycelial mat laden with mature oospores.

2.2.7 Oospore isolation assay

Phytophthora spp. oospores have been reportedly isolated using several methods. Lysing agents, particularly lysing enzymes from *Trichoderma harzianum*, have been used to digest mycelia and sporangia, while leaving thick-walled oospores intact (Lees et al., 2012; Porter et al., 2007). Oospores can also be physically separated from mycelia through sonication and subsequently filtering or decanting the mycelia to obtain a pure oospore suspension (Hood et al., 2014; Widmer, 2010). Alternatively, mycelial mats can be homogenised and mycelial fragments can be removed through centrifugation or filtration (Bi et al., 2012; Dick & Kimberley, 2013; Etxeberria et al., 2011; Krull et al., 2013; Pavón et al., 2008; Williams, 2015). In a number of studies, a combination of these methods were used (Beakes et al., 1986; Delcán & Brasier, 2001; Flier et al., 2001; Jiang & Erwin, 1990; Medina & Platt, 1999; Pittis & Shattock, 1994; Singh et al., 2004; Xavier et al., 2010).

To prepare *P. agathidicida* isolate 18970 mycelial mats for oospore extraction, the incubation liquid was poured off from each Petri plate and a flame sterilised scalpel was used to separate mycelia from the agar plug. The mycelial mat was transferred into a 15 mL Falcon tube with 5 mL sterile MQ^+, which were assigned one of the following treatments:

1. Addition of 5 mL Glucanex (5 mg/mL), a lysing enzyme from *T. harzianum*, followed by a 48-hour incubation at 4°C. Any persistent, visible mycelial was then removed with flame-sterilised

forceps, and oospores were pelleted by centrifuging at 3,220 x ***g*** for 20 minutes. Oospores were washed a total of three times with 5 mL MQ^+ to completely removing the lysing solution, centrifuging between washes (Porter et al., 2007).

2. Homogenisation of the mycelial using a T 25 IKA ULTRA-TURRAX® set at 6,000 RPM for 30 seconds.

3. Sonication of the tubes using a Sonorex Digitec (BANDELIN, Berlin, Germany) at 50% for 60 seconds.

4. Homogenisation followed by the addition of the lysing solution.

5. Sonication followed by the addition of the lysing solution.

6. Homogenisation followed by sonication.

7. Homogenisation, sonication, and then the addition of the lysing solution.

After the final treatment had been applied, the oospores were pelleted by centrifuging at 3,220 x ***g*** for 20 minutes and re-suspended in 1 mL MQ^+.

Three replicates from each treatment were assessed via microscopy, in which 20 µL of each solution was loaded onto a microscope slide and covered with a coverslip. Samples were observed at 40x magnification and photographed with an Olympus DP74 microscope digital camera. The number of oospores, both intact and sheared, and mycelial fragments were recorded for four microscope fields.

2.2.8 Oospore viability assay

Based on the results from the oospore isolation assay (see Section 2.3.1), oospores from *P. agathidicida* isolate ICMP 18970 were isolated through the digestion of mycelia using Glucanex (5 mg/mL). While older protocols for oospore suspensions suggest that the spores are viable for one to two weeks when stored at 4°C (Beakes et al., 1986; Jiang & Erwin, 1990; Sutherland & Cohen, 1983), more recent studies call for the suspensions to be used within 24 hours (Etxeberria et al., 2011; Xavier et al., 2010). Thus, in this study, isolated oospores were used within 24 hours.

Oospore viability was determined through exposing 1 mL oospore suspensions to a variety of different temperatures — room temperature (22°C), 50°C, 75°C — for four hours as well as autoclaving the spore solutions at 121°C for 20 minutes (Dick & Kimberley, 2013; Williams, 2015). All treatments were applied in triplicate. After the treatments had been applied, the oospores were stored at 4°C. Some studies suggest that these samples should be stored for up to 14 days, which would theoretically allow spores that are slow to respond to the treatment to die prior to assessing viability (Dick & Kimberley,

2013). However, it has been more recently shown that there is no detectable difference in viability between heat-treated spores analysed immediately post-treatment with MTT compared to those stored at 4°C for two weeks (Williams, 2015). Thus, it appears that spores affected by heat treatment die rapidly and there is no need for prolonged post-treatment storage. Thus, oospore suspensions were only stored at 4°C overnight after heat exposure, after which their viability was assessed using one of the below methods.

2.2.8.1 Germination

Phytophthora agathidicida oospores have previously been germinated in water on concave microscope slides at room temperature (Williams, 2015). As previously described, there are a variety of incubation media and liquids that have been used successfully to induce oospore germination, and thus, selective agar was used alongside water as a germination media.

Immediately prior to dividing the *P. agathidicida* isolate ICMP 18970 oospore suspensions into different germination treatments, the solution was vortexed to ensure that the oospores had not settled at the bottom of the tube. Spores designed for water germination were transferred either to a 2 mL Eppendorf tube containing 0.5 mL MQ^{+} or into a concave microscope slide, which was then covered with a cover slip and sealed with clear nail polish to prevent the sample from drying out. Selective agar plates were prepared by dividing the agar into three, equal sections, adding 50 µL oospore suspension to the centre of each agar section, and sealing the Petri dish with Parafilm. All samples were stored in the dark at room temperature.

After 2, 4, and 6 weeks, samples were assessed for signs of germination. If a germinated spore was observed, the germination rate was calculated by observing 100 spores using an Olympus BX41 microscope at 40x magnification and determining the ratio of germinated to non-germinated spores. For spores on selective media, samples were assessed using a modified agar block smear preparation (Woo et al., 2010). Briefly, a 10 mm agar core was mounted on a microscope slide and stained with one drop of lactophenol cotton blue, which is a common method for observing *Phytophthora* spores (Erwin & Ribeiro, 1996). The slide was air-dried overnight in a biological safety hood to allow the agar to compress.

At the final time point, samples were observed using an Olympus DP74 microscope digital camera and photographs were taken and analysed.

2.2.8.2 Thiazolyl tetrazolium bromide (MTT) staining

There are three common parameters that are altered in MTT viability assays — concentration of the stain, solvent used to dissolve MTT, and duration of incubation.

MTT is generally added to spore suspensions to yield a final concentration of either 0.05% (Beakes et al., 1986; Dick & Kimberley, 2013; Hood et al., 2014; Jiang & Erwin, 1990; Medina & Platt, 1999) or 0.1% (Etxeberria et al., 2011; McCarren et al., 2009; Williams, 2015). The solution can be prepared in MQ^+ (Beakes et al., 1986; Sutherland & Cohen, 1983), 1 mM potassium phosphate buffer at either pH 3.6 (Medina & Platt, 1999) or 6.3 (Delcán & Brasier, 2001; Etxeberria et al., 2011; Jiang & Erwin, 1990), or 2.8 mM cobalt chloride (McCarren et al., 2009). It has been suggested that the use of potassium phosphate buffer is necessary to stain active oospores blue (Jiang & Erwin, 1990), however, some oospores exposed to lethal conditions have also stained this colour (Etxeberria et al., 2011), Thus, there is persistent ambiguity between colour expression and spore viability. Finally, while most MTT assays recommend that samples are incubated at 35°C for 48 hours, it has been suggested that this can lead to overstaining and increased prevalence of black spores (Etxeberria et al., 2011).

In this assay, 0.1% MTT was prepared using either MQ^+ or 1 mM potassium phosphate buffer (pH 6.3). The MTT solution was added to the *P. agathidicida* isolate ICMP 18970 oospore suspensions at a 1:1 ratio to yield a final MTT concentration of 0.05%. Samples were heated to 35°C using a heating block for 72 hours, with subsamples removed and analysed every 24 hours. For each sample, 100 spores were observed and photographed at 40x using an Olympus DP74 microscope digital camera.

A representative subset of 150 images of stained oospores were also processed using Streams (Nokes, 2021), a program primarily designed for particle detection. Raw images were processed through a filter pipeline, in which green colour values were subtracted from blue. A particle identifier was set up to detect ooplasts that registered a colour intensity value of at least 15, had a maximum aspect ratio of 2, and were between 30 and 120 pixels in diameter.

Furthermore, spectrophotometry was used for MTT viability assessments. As formazan products are insoluble in water, a MTT solubilisation solution was prepared, which contained 40% (v/v) dimethylformamide, 2% (v/v) glacial acetic acid, and 16% (wt/v) sodium dodecyl sulphate (Riss et al., 2013). Additionally, DMSO was used as an alternative formazan solubilisation solvent (Stockert et al., 2018). Solubilisation solutions were added at a 1:1 ratio to oospore suspensions, shaken for 15 minutes at medium speed, and then analysed with a Multiskan GO (Thermo Fisher Scientific, Vantaa, Finland) reading absorbance at 570 nm.

2.2.8.3 Plasmolysis

As previously mentioned in Section 2.1.2.2, plasmolysis can be induced using either sucrose or NaCl solutions at different concentrations. This assay opted to use NaCl to induce plasmolysis in oospores, as it was more frequently used in *Phytophthora* plasmolysis studies. Furthermore, a relatively concentrated solution, 3M, was used, as this has been reported to induce higher plasmolysis rates (Etxeberria et al., 2011; Jiang & Erwin, 1990).

A 4 M NaCl solution was prepared in sterile MQ^{+} and added to each *P. agathidicida* isolate ICMP 18970 oospore suspension at a 3:1 ratio, which yielded a final concentration of 3M NaCl. The samples were incubated for 60 minutes after which a subsample was removed for microscopy. For each sample, 100 spores were observed and photographer at 40x using an Olympus DP74 microscope digital camera.

2.2.9 Statistical analysis

2.2.9.1 Oospore isolation

The results were analysed using a one-way Analysis of Variance (ANOVA), wherein the treatments (homogenisation, sonication, addition of lysing agent, etc.) were designated as levels and treated as the independent value and the count data (sum of four microscope fields for each replicate) was assigned as the dependent variable. Counts for sheared oospores were not statistically analysed, as they were not considered to be an independent observation. If the raw data was not normally distributed, as determined through the Shapiro-Wilk test (Shapiro & Wilk, 1965), a natural log or cube root transformation was applied. If count data was significantly related to the isolation treatment applied ($p < 0.05$), a Tukey's Honestly Significant Differences (HSD) test was applied. All analyses were carried out on R v3.6.0 (R Core Team, 2013).

2.2.9.2 Oospore viability

The MTT results were analysed using a series of one-way ANOVAs to test the effect of incubation period (e.g., 24, 48, and 72 hours) on stain intensity as well as the effect of heat on spore viability. Data was normalised using a square root or cube root transformation, if necessary. If the ANOVA showed a significant treatment effect ($p < 0.05$), a Tukey's HSD test was applied. For datasets that could not be normalised, no further analysis was conducted.

2.3 Results

2.3.1 Optimising oospore isolation

All seven treatments yielded isolated oospores, although there was considerable variation in the number of oospores isolated as well as the presence of sheared spores and mycelial fragments (Figure 2.8 and Figure 2.9). Notably, on its own, sonication yielded the fewest number of isolated oospores, treatments that included homogenisation resulted in sheared oospores, and despite the use of a lysing agent, all enzyme treated samples had persistent mycelial fragments (Figure 2.8 and Figure 2.9). It is worth noting, however, that mycelia exposed to Glucanex appeared morphologically different to those produced by homogenisation, both in terms of the length of the fragments and the structural integrity of the hyphae. Based on the high number of oospores and relatively low hyphal contamination in the enzyme treatment, this method was used in subsequent assays.

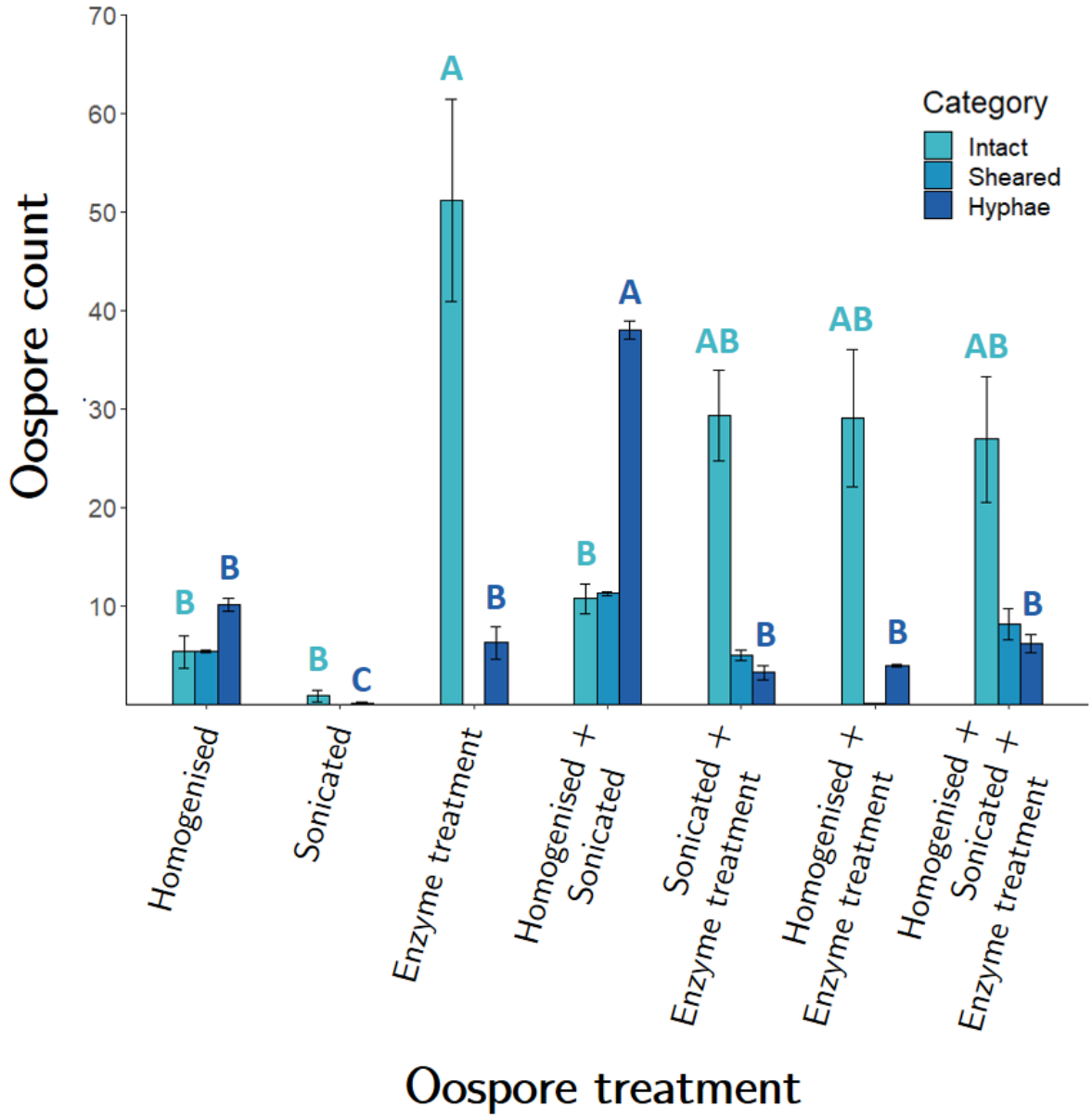

Figure 2.8: Differences in the number of intact and sheared oospores as well as hyphal fragments of *Phytophthora agathidicida* isolate ICMP 18970 produced by each oospore isolation treatment
Error bars depict the standard error based on three replicates from the same treatment. Count values that share the same letter within the same category (e.g., intact or hyphae) across different treatments (e.g., homogenised, sonicated, etc.) are not statistically different ($p < 0.05$, Tukey's test). The full Tukey's test output can be found in Table A.1 and Table A.2.

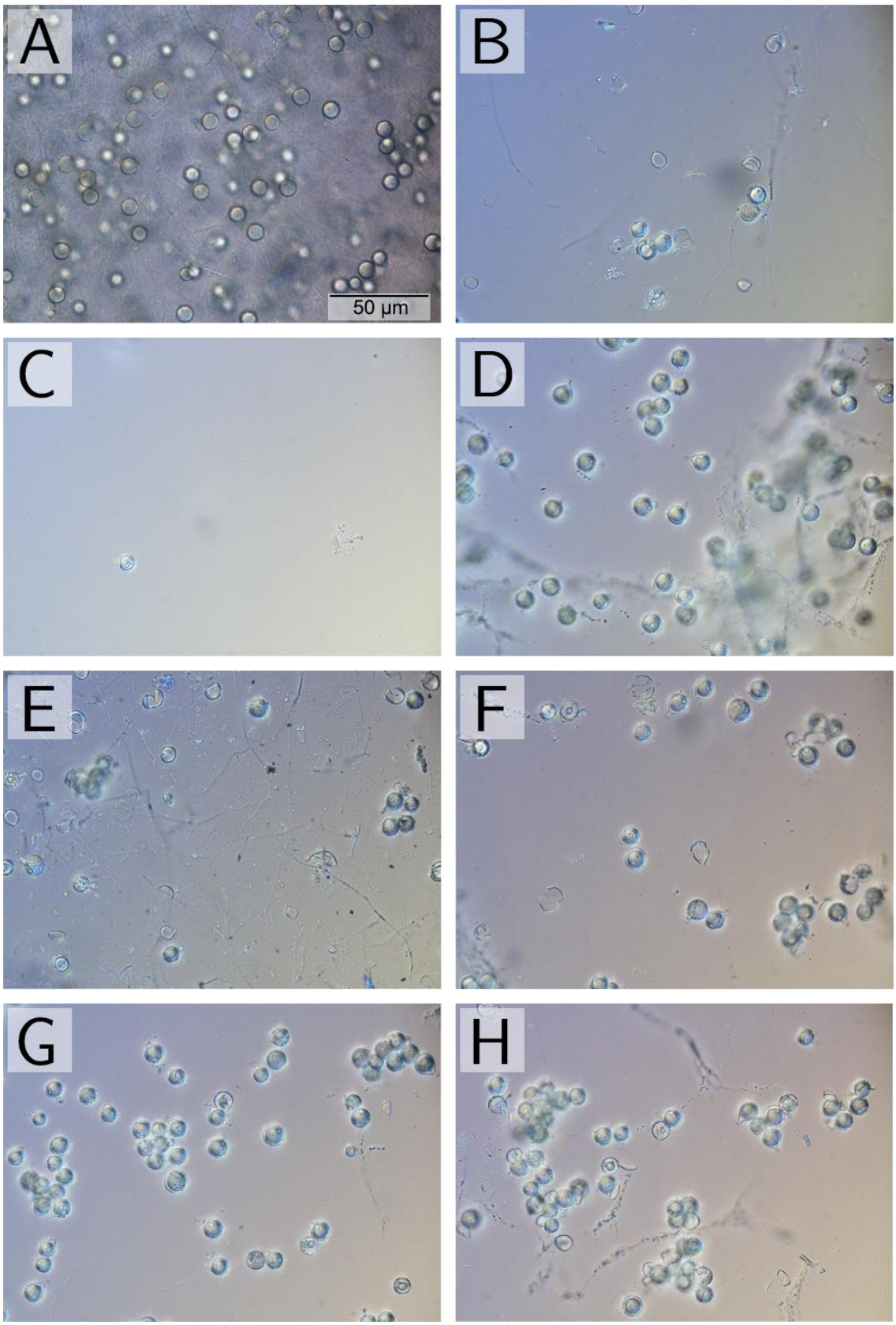

Figure 2.9: Appearance of *Phytophtora agathidicida* isolate ICMP 18970 oospore suspensions using different oospore isolation methods taken at 20x magnification
A| Untreated mycelial laden with oospores, B| homogenisation, C| sonication, D| enzyme digestion, E| homogenisation and sonication, F| homogenisation and enzyme digestion, G| sonication and enzyme digestion, H| homogenisation, sonication, and enzyme digestion.

2.3.2 Germination rates of *P. agathidicida* oospores

Oospore were classified as germinated if the ooplast was no longer intact and freshly produced mycelia was visible (Figure 2.10).

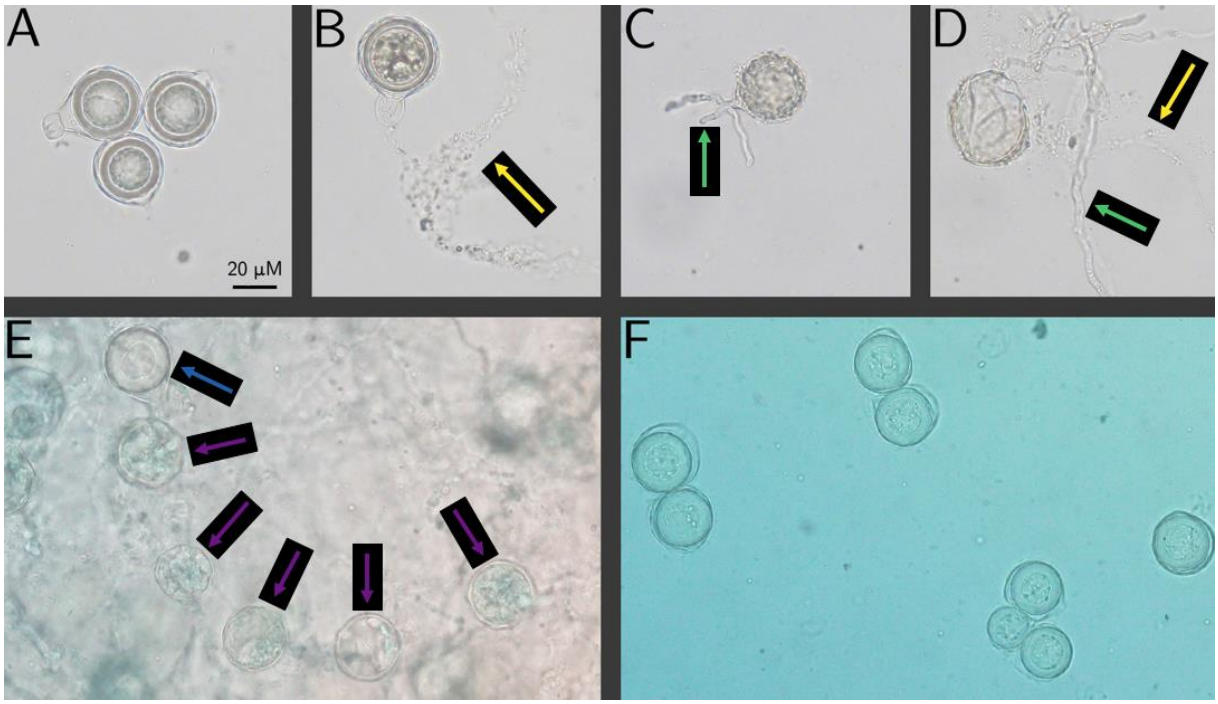

Figure 2.10: Germinated and non-germinated *Phytophthora agathidicida* oospores
Images a-d are from the water incubation while images e and f were germinated on selective media. a| Non-germinated spores, b| a non-germinated spore with persistent mycelia from the enzyme treatment, c| a germinated oospore, d| a germinated oospore, with arrows highlighting persistent (yellow) and fresh (green) mycelia, e| oospores on agar media, with arrows highlighting non-germinated spores (blue) and germinated oospores (purple), f| non-germinated oospores that were heat treated.

Germination was only observed in samples treated at 22°C and the germination rates differed between incubation media (Table 2.2). Due to the widespread germination failure in samples treated above 22°C, this dataset was not normalised and no statistical analysis was performed. Interestingly, the storage method of spores also appears to have affected germination rates, as oospores stored in water in 2 mL Eppendorf tubes had a higher germination rate compared to those incubated on microscope slides. Whereas germinated oospores were only observed after four weeks when incubated in MQ^{+} water, visible mycelium was observed after one week on selective agar media (Figure A.1).

Table 2.2: Germination rates of heat-treated *Phytophthora agathidicida* oospores on different media
Variance is reported as standard error based on three replicates.

	Temperature treatment			
Incubation media	22°C	50°C	75°C	Autoclaved
Selective agar	74 ± 6.9%	0%	0%	0%
MQ+ water (2 mL Eppendorf)	6 ± 2.9%	0%	0%	0%
MQ+ water (Microscope slide)	0%	0%	0%	0%

2.3.3 Staining of *P. agathidicida* oospores with MTT

MTT stained spores varying degrees of tints and shades of purple, with no blue spores observed regardless of the solvent used to dissolve the MTT. Based on the descriptions provided by previous studies (Table 2.1), the following viability designations were made — clear (1) and black (6) oospores were non-viable, pink oospores (2 and 3) were dormant, and purple oospores (4 and 5) were active (Figure 2.11). These viability assignments most closely reflect that of a study conducted by Dick & Kimberly (2013), which also studied *P. agathidicida*.

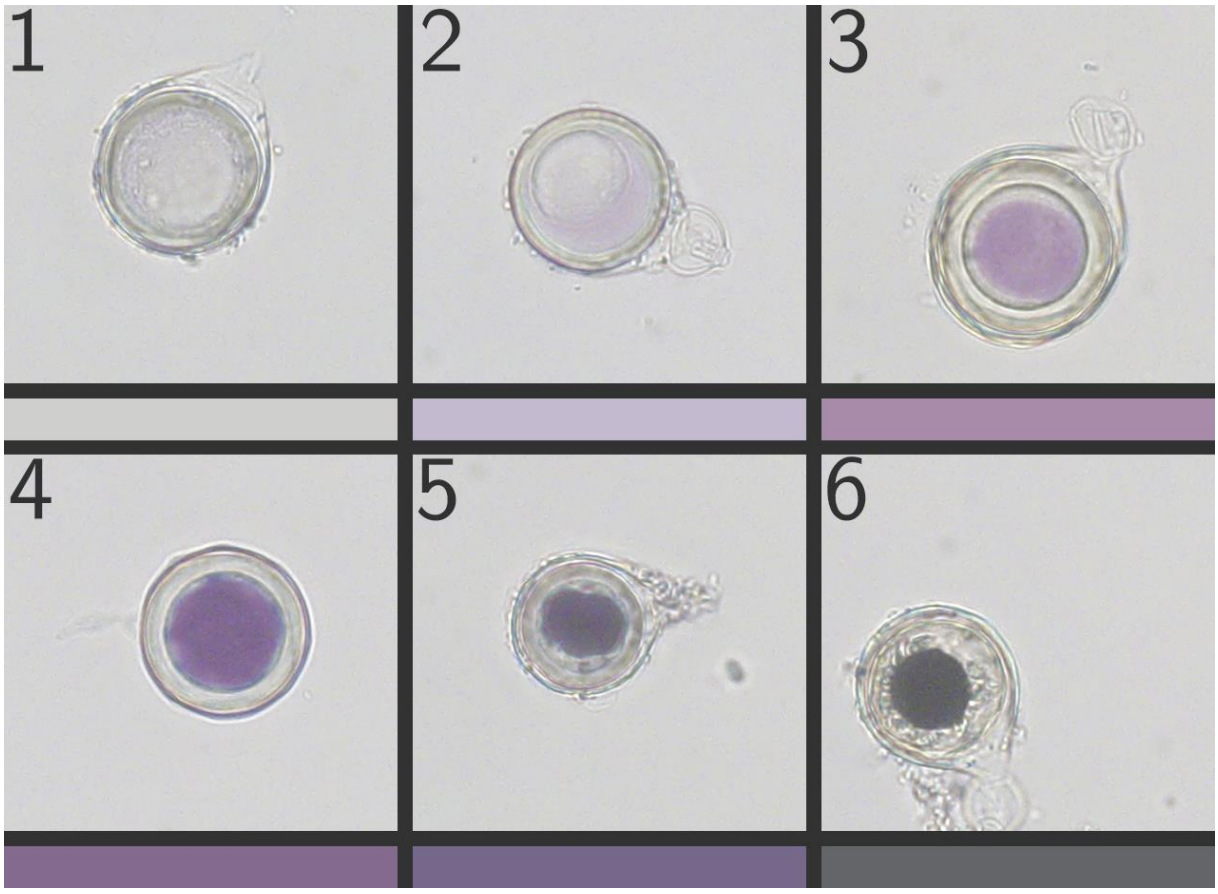

Figure 2.11: Variation in *Phytophthora agathidicida* oospore vital staining with the use of thiazolyl tetrazolium bromide (MTT)
Beneath each photograph is a representative colour swatch taken directly from the ooplast.

Stain development, as provided in Figure 2.11, was monitored in three replicate controls (e.g., non-heat treated) over a 72-hour period (Figure A.2). There were decreasing trends of lighter stain intensities (levels 1 and 2) and increasing trends of darker stain intensities (levels 4, 5, and 6) over time, however, none of these shifts were statistically significant. When classifying oospores viability (i.e., active, dormant, nonviable) in these samples, there were significantly higher rates of oospores classified as active when oospores were exposed to MTT for at least 48 hours (Figure A.3). As no previous study appears to use a 72-hour incubation period with MTT, further vital staining with MTT used a 48-hour incubation period.

When oospores were heat treated, the MTT viability assay showed a significant decrease in active spores in heat treated spores (Figure 2.12). Interestingly, if the sum of active and dormant oospores is considered to be "viable" spores, there is not a significant difference between viable spores in the control (22°C) and 50°C heat treatment. Rather, it appears that a large proportion of viable spores treated at 50°C were rendered dormant in comparison to the control. While the number of nonviable spores present at temperatures of 75°C and higher increased, this dataset could not be normalised, and thus further statistical analysis was not conducted.

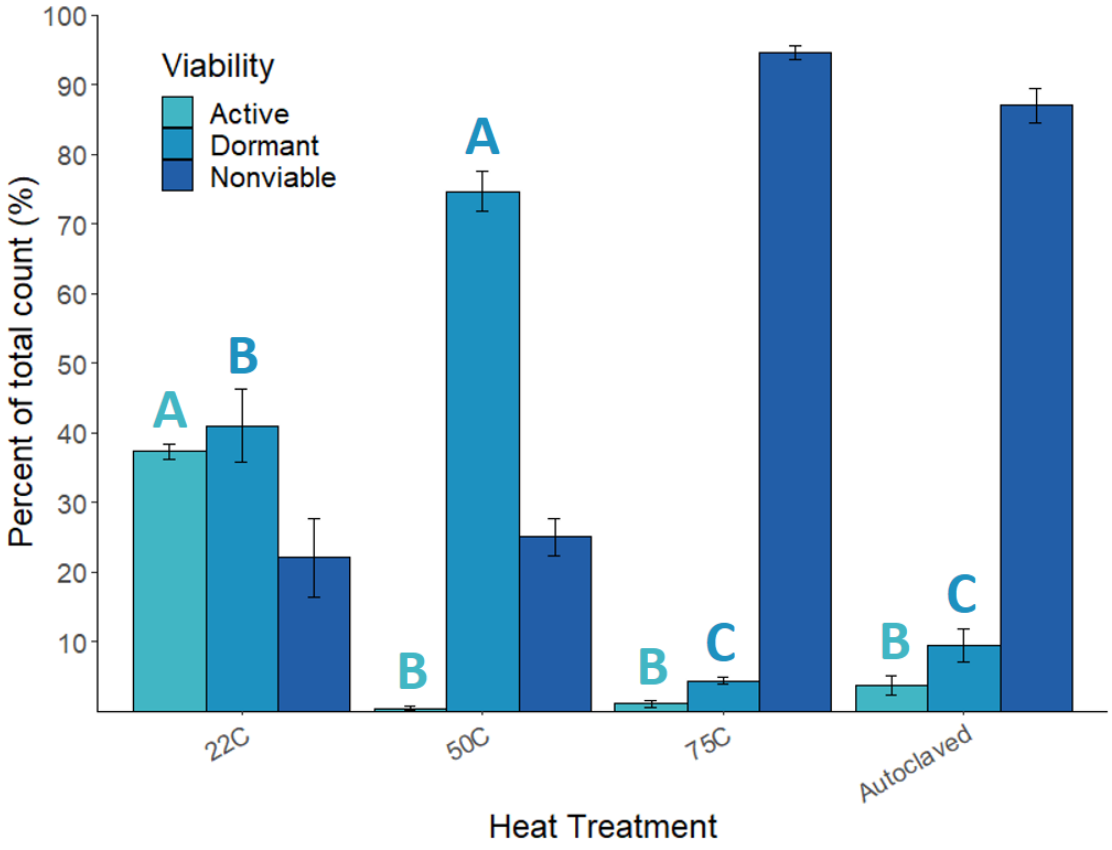

Figure 2.12: Viability of *Phytophthora agathidicida* oospores exposed to heat as determined through thiazolyl tetrazolium bromide (MTT) vital staining
Error bars depict the standard error based on three replicates. Percent values that share the same letter within the same category (e.g., active or dormant) across different treatments (e.g., 22°C, 50°C, etc.) are not statistically different ($p < 0.05$, Tukey's test).

The rate of false positives with MTT staining was determined by calculating the number of stained spores, either dormant or active, in the autoclaved treatment. This yielded a false positive rate of 13 ± 2.5%, which is similar to previous studies (Medina & Platt, 1999; Singh et al., 2004). The rate of overstaining, as suggested by Etxeberria et al. (2011), was calculated by determining the proportion of black spores in control samples (22°C). Overstaining occurred at extremely low rates (1.7 ± 0.72%).

2.3.4 Detection of formazans with spectrophotometry

Viability of MTT stained oospores could not be determined with the use of a spectrophotometer. Neither the addition of a solubilisation solution nor DMSO produced a reading at 570 nm that could be distinguished from controls, which were MTT treated oospores that received DI water rather than a solution designed to dissolve the formazans.

2.3.5 Detection of stained oospores with Streams, an image processing software

The Streams filter pipeline and particle detection program (Figure 2.13) was successfully able to detect viable oospores (Figure 2.14a, c, and g) and ignore non-viable oospores (Figure 2.14a and e) with an 86% agreement rate with visual assessments. The majority of disagreements arose from instances in which lightly stained oospores were identified visually, but did not meet the threshold for particle identification (Figure 2.14f), although on occasion, oospores stained dark purple were also misidentified as black (Figure 2.14b). The program only falsely identified viable oospores 1% of the time, which occurred when pigmented mycelia was present (Figure 2.14h). From start to finish, the application of the filter pipeline and particle detection analysis took 35 seconds.

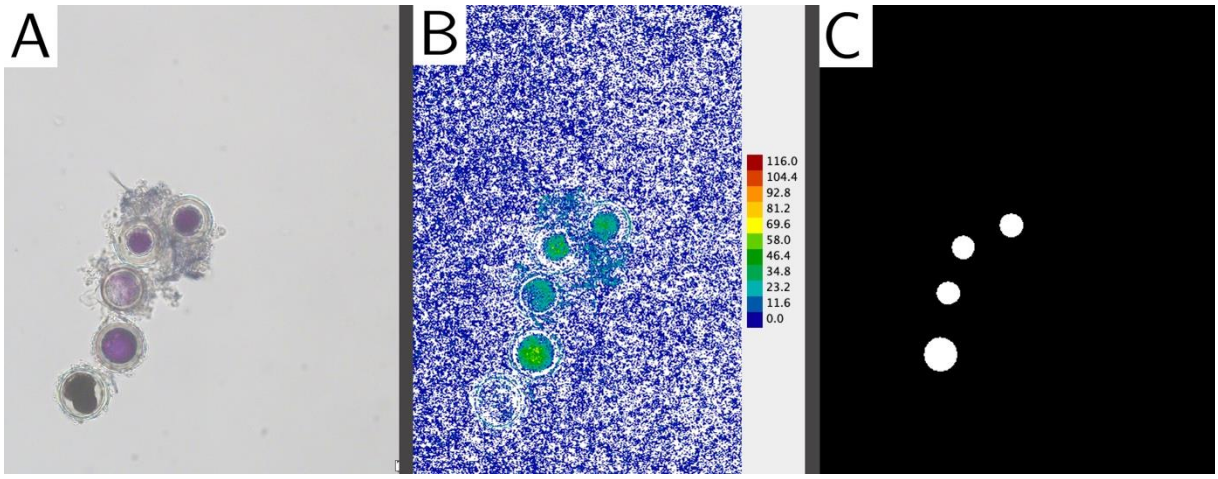

Figure 2.13: Image processing and viable spore detection with the software Streams
a| Raw image showing four purple stained (viable) and one black stained (non-viable) oospores of *Phytophthora agathidicida*. b| Processed image after the filter pipeline sequence was applied. The relative intensity threshold is shown on the right-hand side. c| The particle detection program identified four particles based on the criteria outlined in Section 2.2.8.2.

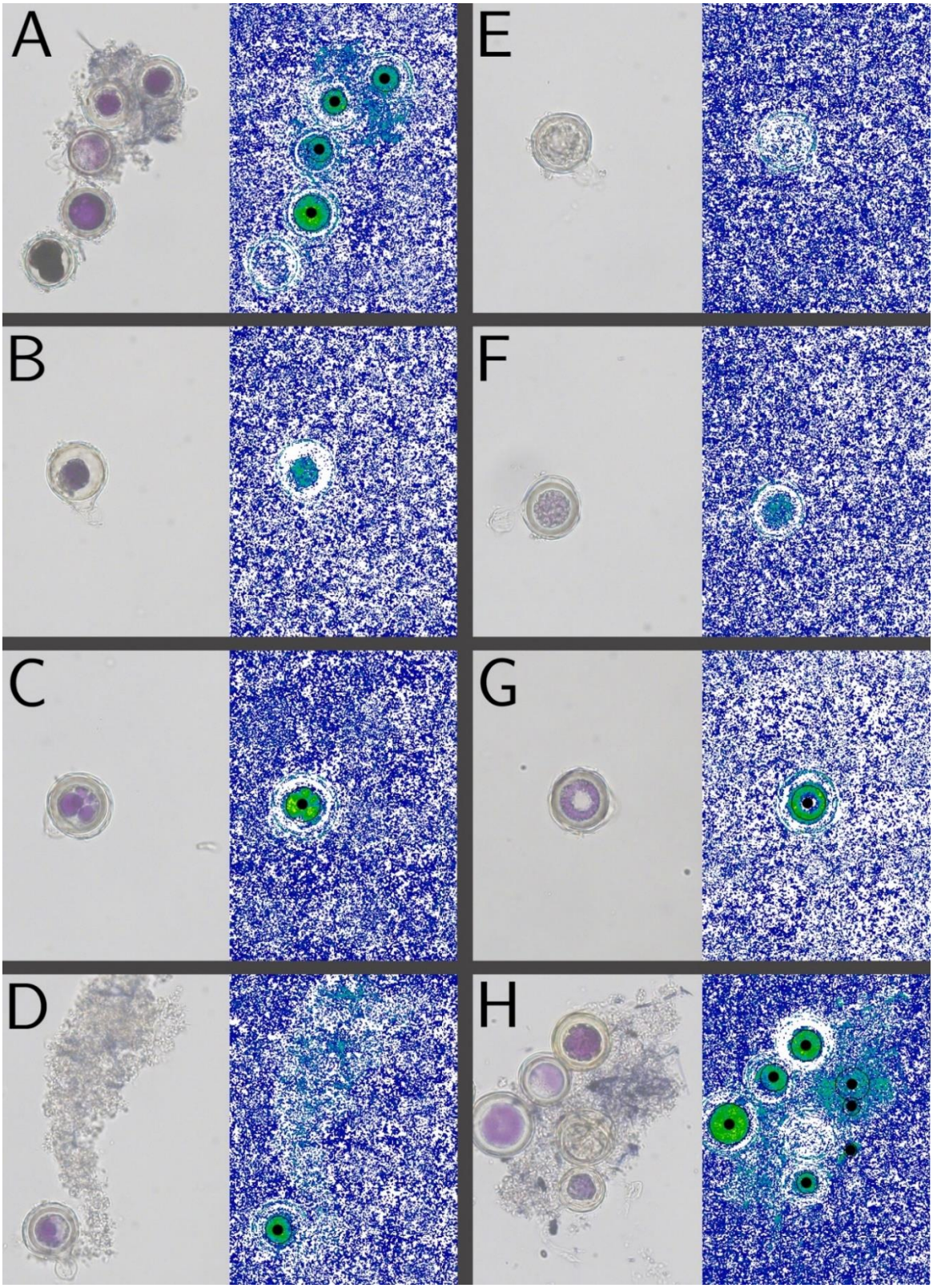

Figure 2.14: Accuracy of the particle detection program Streams on thiazolyl tetrazolium bromide (MTT) stained *Phytophthora agathidicida* oospores

For each image set, the left-hand image is the raw photograph whereas the right-hand image shows the applied filter and any identified viable spores with a black dot. The program correctly ignores a| black stained and e| clear oospores as well as d| non-pigmented mycelia. On occasion it does not detect b| dark or f| light stained oospores or h| erroneously detects mycelia. The program successfully c| identifies one viable spore when multiple nuclei are present in an ooplast; and g| detects irregularly shaped or stained ooplasts.

2.3.6 Plasmolysis of *P. agathidicida* oospores

Plasmolysis was not induced in heat-treated *P. agathidicida* oospores. What's more, there was considerable variation in ooplast appearance in control samples that were not heat-treated nor exposed to the hypertonic solution (Figure 2.15). This included oospores that possessed similar characteristics to plasmolysised oospores identified in previous studies (Figure 2.5b and c), in which the ooplast was partially retracted.

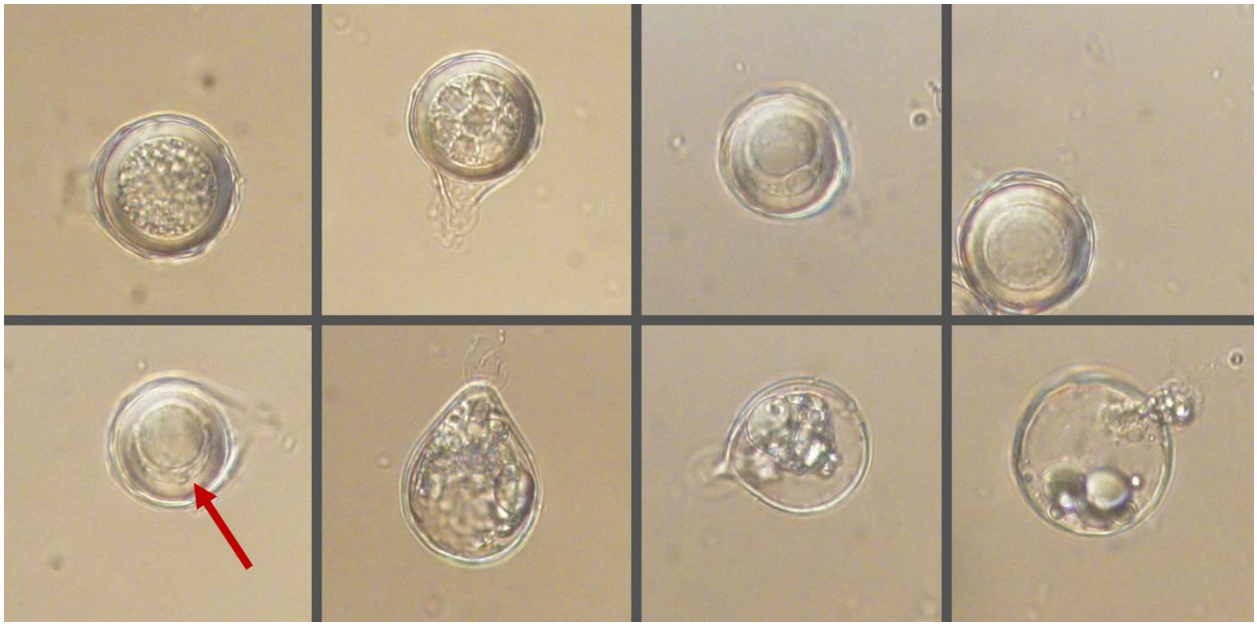

Figure 2.15: Variation in *Phytophthora agathidicida* ooplast appearance in non-heat treated, non-plasmolysised oospores
A red arrow denotes an oospore that potentially resembles a plasmolysised oospore based on imagery provided by previous studies, in that there is observable retraction of the ooplast from the thick, inner oospore wall.

2.4 Discussion

2.4.1 Isolation of *P. agathidicida* oospores

In this study, *P. agathidicida* oospores were more effectively isolated with the use of a lysing enzyme. However, based on many other published methods (see Section 2.2.7) it is likely that the failure of homogenisation and sonication to isolate oospores in this experiment was due to the constraints of the instruments available, rather than intrinsic faults in these techniques. Additionally, the specifications of homogenisers and sonicators used to isolate oospores are not frequently published, whereas the exact lysis product are generally stated, which made it easier to successfully replicate enzyme lysis studies. The sonicator used in this study was an ultrasonic bath, whereas a sonication probe might more effectively disrupt oospores on mycelia. While the T 25 IKA ULTRA-TURRAX® was not appropriate for this assay, due to the shearing of spores, it is very likely that using a different homogeniser, such as the ULTRA-TURRAX® Tube Drive (Dick & Kimberley, 2013; Williams, 2015),

applying an alternative dispersing attachment, or changing the homogenisation time period would achieve different results. Furthermore, based on the mycelial contamination in samples treated with a homogeniser, it seems that repeated centrifugation washes are not sufficient to remove mycelia from the oospore suspensions. Thus, a filtration step using mesh with a pore size between 20 and 25 µm, based on the average *P. agathidicida* oospore width (Weir et al., 2015), should be considered for future assays.

2.4.2 Persistent issues with oospore viability assays

2.4.2.1 Difficulties with oospore germination

Germination of *P. agathidicida* oospores occurred in MQ^{+} and on selective agar media, with significantly higher germination occurring on agar. That being said, as oospores on selective media germinated to produce dense hyphae after one week, it was not always possible to identify the newly produced mycelia, distinguish aborted oospores lacking an ooplast from fully germinated oospores devoid of protoplasm, and determine whether non-germinated oospores originated from the heat-treated sample or if they had been newly produced from the fresh mycelial growth. Thus, it would be beneficial to observe *P. agathidicida* oospores on selective agar media again using a shorter time interval to better approximate germination rates as well as provide a more comprehensive visual guide for germination of *P. agathidicida* oospores. The latter would be particularly beneficial, as the appearance of germinated *P. agathidicida* oospores appears to have varied between this study and one that was conducted previously (Figure 2.16). Specifically, this study found that germinated oospores consistently lacked a thick inner wall, which would have resulted from the digestion of glucans during germination (Erwin & Ribeiro, 1996). Conversely, another *P. agathidicida* study appears to show germinated oospores with fully intact ooplasts, however this may be an artefact of the image quality.

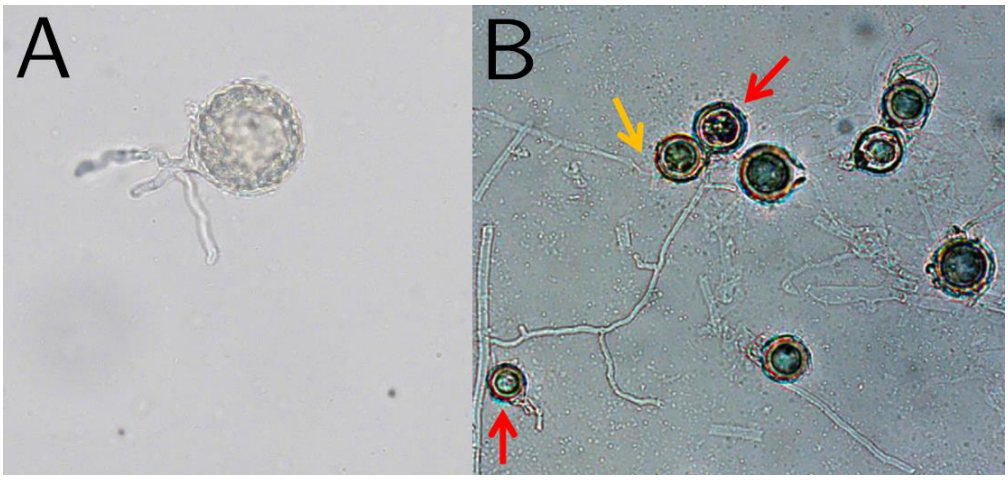

Figure 2.16: Morphology of germinated *Phytophthora agathidicida* oospores between two studies
A| Germinated oospore from Thurston, (2021), B| arrows indicating germinated oospores with freshly produced mycelia from Williams, (2015).

Germination was only observed in samples treated at 22°C, however, based on a previous study (Williams, 2015) it was expected that germination would also occur at 50 and 75°C, albeit at much lower rates. It is not entirely clear why germination was not observed in heat-treated oospores, but it could be linked to non-optimised incubation conditions. This could potentially be resolved by altering the incubation media — for example, diluted pond water (Xavier et al., 2010) —, increasing the light availability for germination, or adding a chemical stimulant for germination, such as potassium permanganate ($KMnO_4$) (Ann & Ko, 1988; Guo & Ko, 1994).

2.4.2.2 Inconsistencies with MTT staining and classification

The trend of increasing stain intensity across 72 hours was not significant (Figure A.2). Thus, this study did not find a correlation between the presence of black spores and the so-called "overstaining" phenomenon. However, this does not inherently conflict with previous findings (Etxeberria et al., 2011), as this study used a lower concentration of MTT to stain oospores.

Previous studies that have assessed heat-treated *P. agathidicida* oospore viability through vital staining have reported contrasting results, both with regard to the number of viable oospores in controls as well as the viability of oospores treated at temperatures ranging from 50 to 70°C (Dick & Kimberley, 2013; Williams, 2015). It is possible that these differences stem directly from how stained spores were classified, particularly the distinction between active, dormant, and the sum of the two, viable. To this point, the proportion of active and viable spores documented in this study were compared with the two previously reported datasets (Figure 2.17). To validate this theory, however, the actual photographs of stained spores would need to be compared.

Some of the inconsistencies of this assay may be alleviated through the use of more comprehensive classification keys. As previously documented (Table 2.1, Figure 2.4), there is variation in both terminology and stain intensity reported in the literature. Furthermore, many studies only provide imagery for a clear and highly stained, viable spore, without documenting the range of MTT staining observed or the colour range for dormant spores. The use of a spectrophotometer could circumnavigate the lack of visual aids provided in the literature. While this study was not able to effectively detect oospore viability using a spectrophotometer, there are a few assay parameters that could be adjusted to yield more successful outcomes. Specifically, the concentration of oospores per sample could be increased (Williams, 2015), alternative solubilisation solutions could be used, such as dimethylformamide and acidified isopropanol (Riss et al., 2013), or formazans could be dissolved under different conditions, such as 37°C (Kamiloglu et al., 2020).

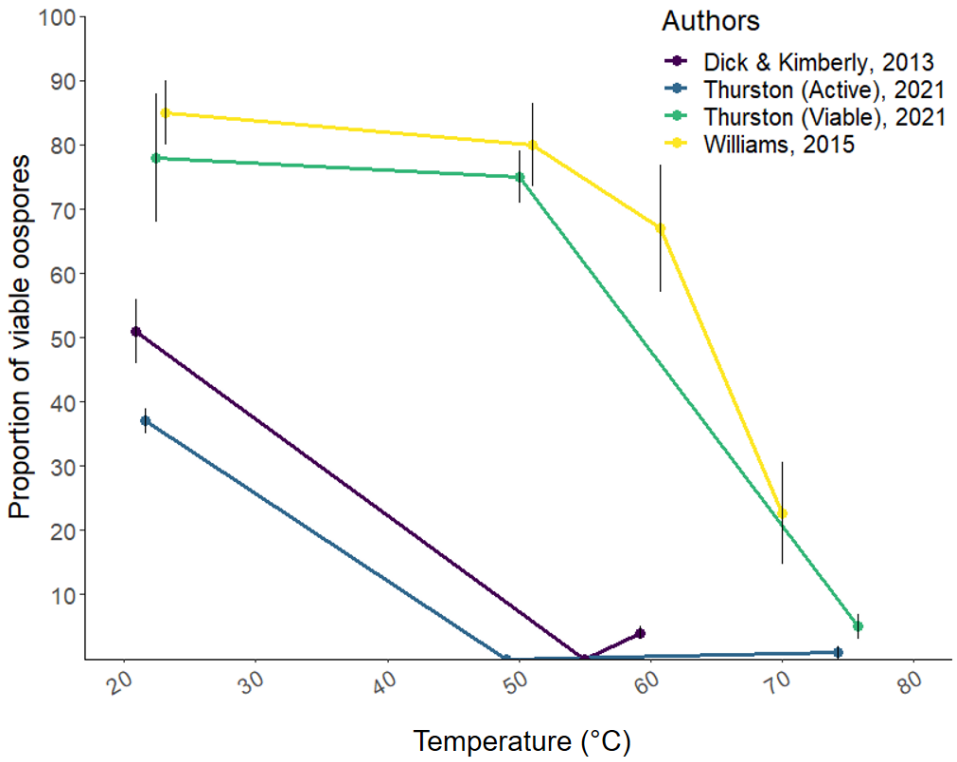

Figure 2.17: Proportion of viable, heat-treated *Phytophthora agathidicida* oospores from three different studies
The Thurston, (2021) dataset has been distinguished into two categories — active and viable, the latter of which is the sum of active and dormant spores. For the Dick & Kimberly (2013) and Williams (2015) datasets, each data point and error range is an approximation from the graphs provided in their respective reports.

Viability assessments of spores and cells using non-fluorescent based imaging software is a relatively new field with few applications (Gasparini et al., 2017; Lee et al., 2014; Sediq et al., 2018). Based on the available literature, this appears to be the first application of Streams to determine cell viability, and only the second study to use imaging software to assess viability in an MTT assay (Lee et al., 2014). The agreement between visual assessments and Streams particle detection was approximately 86% in this study. However, most false negatives arose from lightly stained oospores (Figure 2.11, classifications 1 and 2), which may have been incorrectly distinguished from clear oospores in visual assessments due to assessor bias. Thus, it is very likely that the false negative detection rate using Streams is well below 15%. To further validate this hypothesis, it would be pertinent to have additional, trained assessors evaluate MTT treated oospores for viability. This would allow for a better understanding of how the detection assessments made by the software compares to a range of subjective, visual-based assessments. Furthermore, properly ascertaining the correlation between germination rates and MTT staining would allow for a better understanding of the relationship between stain colour or intensity and actual spore viability. This could be used to better calibrate the sequential filter pipeline and detection protocol used in Streams.

While the Streams parameters used in this study were able to identify stained spores, studies seeking to validate or adapt this method would likely need to optimise the Streams protocol, as detection is

highly influenced by the light level and consistency of the microscopy images, the presence of cellular debris, and the colour register of the camera used.

2.4.2.3 Inability to induce plasmolysis

This study was not able to induce plasmolysis in *P. agathidicida* oospores, which has been reported previously for other *Phytophthora* spp. (Porter et al., 2007). It is possible that the molarity of NaCl used was not sufficiently hypertonic enough to induce plasmolysis within the given time-frame of one hour. However, due to the variation in oospore appearance (Figure 2.15), oospore viability as determined by plasmolysis could still be relatively subjective. It is possible that the variation observed here could be due to the presence of immature and developing oospores, in which case extending the duration of oospore production and maturity from six to eight weeks could result in a higher proportion of fully mature spores with less ooplast variation.

2.4.3 Methods comparison

There was agreement between germination rates of oospores considered "viable" in the MTT assay and direct germination of oospores on selective agar media for samples treated at 22°C, 78 ± 5.5% and 74 ± 6.9% respectively. As germination was not observed for heat-treated oospores on agar, it is not possible to fully compare the accuracy of MTT vital staining with oospore viability.

2.5 Conclusions

Phytophthora agathidicida oospores were most readily isolated using a lysis agent, which had not been previously used for this pathogen. As other studies that have worked with *P. agathidicida* oospores did not report sheared oospores, it is also possible that using the ULTRA-TURRAX® Tube Drive homogeniser followed by filtration would also be appropriate for oospore isolation.

While this study attempted to optimise and compare methods for determining *Phytophthora agathidicida* oospore viability, it encountered similar issues that have been previously reported, including problems with germination conditions (Jayasekera et al., 2007; McCarren et al., 2009), subjectivity of MTT vital staining (Etxeberria et al., 2011; Williams, 2015), and failure to properly induce plasmolysis (Porter et al., 2007). That being said, this study has highlighted future avenues for assay optimisation for all three methods, has provided a clear MTT staining key for *P. agathidicida*, and was successful in applying a new software to assess spore viability with vital staining that reduces the assay's subjectivity.

Chapter 3:

Inhibition of *Phytophthora agathidicida* with commercially available fungicides and essential oils

3.1 Introduction

Diseases caused by oomycetes are in part remedied by chemical treatments (Derevnina et al., 2016; Pscheidt, 2021). Unfortunately, oomycetes are largely unaffected by most commercial fungicides (Gisi & Sierotzki, 2015). This stems from the fact that agricultural fungicides typically target pathways that are specific to true fungi, such as those involving chitin and ergosterol production (Hardham, 2005; Lawrence et al., 2019). To date, there have been 17 chemical groups identified that affect oomycetes (Gisi & Sierotzki, 2015; Pasteris et al., 2016), which include single site inhibitors (e.g., phenylamides and carboxylic acid amides), multi-site inhibitors (e.g., dithiocarbamates and copper formulations), and chemicals for which the exact mode of action is unknown (e.g., fosetyl-Al and fluopicolide) (Gisi & Sierotzki, 2015). Of these, phosphonate- and phenylamide-based compounds are the most common chemical agents used for *Phytophthora* control (Belisle et al., 2019).

3.1.1 Current chemical control of *P. agathidicida*

Chemical control of *P. agathidicida* can be divided into three branches — chemicals that are used to treat kauri dieback, prevent *P. agathidicida* spread, or those in the pipeline for potential application.

3.1.1.1 Chemicals used to treat kauri dieback

Only phosphite and metalaxyl, a phosphonate and phenylamide respectively, have been tested *in vivo* for *P. agathidicida* disease control in kauri. Applications of metalaxyl granules in a glasshouse trial only slightly decreased kauri seedling mortality, whereas injections of phosphite significantly reduced root and canopy disease symptoms (Horner & Hough, 2013). Further phosphite trials in dieback areas have found that phosphite treated kauri had significantly decreased lesion activity and expansion, although high concentrations of phosphite resulted in phytotoxicity symptoms in aboveground biomass (Horner et al., 2015). Phytotoxicity caused by phosphite generally presents as necrosis, defoliation, and chlorosis (Hardy et al., 2001). While foliar damage is typically irreversible, phytotoxicity does not affect new growths (Hardy et al., 2001). To address this issue, an additional study tested different phosphite dosages and found that concentrations as low as 4% provided effective disease control without causing additional damage to the tree (Horner et al., 2019).

While phosphite injections are the only validated treatment for kauri dieback, there are concerns over its long-term usage. Phosphite only acts to mitigate disease symptoms, but does not actually cure infected kauri (Bradshaw et al., 2019). Additionally, the longevity of the treatment efficacy is unknown, and thus, it is not clear how frequently kauri would require re-treatment (Horner et al., 2015). This is further complicated by the fact that long-term usage of phosphite may lead to the development of phosphite resistance in *P. agathidicida* (Bradshaw et al., 2019). Although phosphite resistance has been generally classified as low risk (Fungicide Resistance Action Committee, 2019), it has occurred in *Phytophthora* pathogens before (Dobrowolski et al., 2008; Veena et al., 2010; Wilkinson et al., 2001). There are also cultural concerns over phosphite as a treatment, as some iwi worry that wide-scale applications of phosphite may unintentionally impact other organisms and taonga in kauri forests (Black & Dickie, 2016). Specifically, run-off phosphite may impact the efficacy of rongoā and the availability of kai (Nuttall et al., 2010).

3.1.1.2 Chemicals used to manage kauri dieback spread

Disinfectant stations have been installed on boardwalks throughout and around kauri forests, with the aim of reducing disease spread by human vectors (Ministry of Primary Industries, 2014). These stations disperse TriGene II Advance, which effectively suppresses *P. agathidicida* mycelia growth and zoospore activity, but does not eliminate the viability of oospores (Bellgard et al., 2010a; Dick & Kimberley, 2013; Pau'uvale et al., 2011).

Other disinfectants have been found to more successfully render oospores of *P. agathidicida* nonviable, namely Virkon™ and Janola, however, these disinfectants have limited application due to the fact that they are corrosive to metals and bleach clothing (Bellgard et al., 2010).

3.1.1.3 Chemicals with documented *in vitro* inhibition of *P. agathidicida*

High-throughput chemical screenings have identified a number of compounds that inhibit the growth and viability of *P. agathidicida* and *P. cinnamomi*, another *Phytophthora* pathogen of kauri (Scott & Williams, 2014). These compounds include antibiotics, copper compounds, a quaternary ammonium cation, as well as a range of natural products (Lawrence et al., 2017, 2021). Presently, these chemicals have only been tested *in vitro* against different *P. agathidicida* life stages and still require *in vivo* confirmation.

3.1.2 New fungicidal candidates

Recently, a range of new anti-oomycete fungicides have become commercially available — ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin (Belisle et al., 2019). All four compounds are single site inhibitors with different modes of action to one another and to phosphonate and phenylamide fungicides (Belisle et al., 2019; Hao et al., 2019).

Ethaboxam is a thiazole carboxamide that functions as a β-tubulin inhibitor (Peng et al., 2019). β-tubulin polymerise into microtubules, a key component of the cytoskeleton that plays a central role in nuclear migration (Uchida et al., 2005). Thus, inhibition of β-tubulin leads to disruption of both mitosis and cellular division (Hao et al., 2019). Ethaboxam reportedly acts systematically in plant hosts and provides both preventative and curative control mechanisms (Kim et al., 2004). Furthermore, ethaboxam may have highly specific activity, as one study found that it did not inhibit microtubule formation in *Aspergillus nidulans* and mouse culture cells (Uchida et al., 2005). A field study also found that the control efficacy of ethaboxam remained high over time (Zhang et al., 2005), indicating that it may be at low risk for resistance development. That being said, ethaboxam resistance in *Phytophthora* pathogens has already been induced in the lab (Peng et al., 2019).

Fluopicolide also affects the cytoskeleton, but instead delocalises spectrin-like proteins (Jiang et al., 2015). Based on field trials, fluopicolide appears to have greater potential as a protective fungicide, as opposed to a curative one (Shin et al., 2010). There is disagreement over the risk of resistance development for this fungicide, with some studies reporting it as a "moderately high risk" (Lu et al., 2011; Wu et al., 2020) whereas others classify it as a "low to moderate risk" (Zhang et al., 2005). It is presently classified by the Fungicide Resistance Action Committee as "medium risk" (Fungicide Resistance Action Committee, 2019).

Mandipropamid is a mandelic acid amide that targets the cellulose pathway and consequently impedes cell wall biosynthesis in *Phytophthora* (Blum et al., 2010; Gisi & Sierotzki, 2015). This compound has been reported to be of low to medium risk for resistance development (Fungicide Resistance Action Committee, 2019).

Oxathiapiprolin inhibits a novel oomycete target, an oxysterol binding protein (OSBP) (Pasteris et al., 2016). While the exact function of this protein is not known (Pasteris et al., 2016), OSBP are reportedly involved in cell signalling, lipid metabolism, and both vesicular and non-vesicular trafficking and transportation (Raychaudhuri & Prinz, 2010). This compound can act as either a preventative fungicide by inhibiting zoosporogensis or a curative agent by impeding mycelial growth within plant tissue and inhibiting lesion expansion (Pasteris et al., 2016). Oxathiapiprolin has been classified as having medium to high risk for resistance development (Fungicide Resistance Action Committee, 2019), and oxathiapiprolin resistant *Phytophthora* pathogens have already been generated in the lab (Miao et al., 2016a).

To date, most of the work involving the effect of these four anti-oomycete fungicides in *Phytophthora* has been conducted on mycelia (Table 3.1). Of the four, oxathiapiprolin has been generally identified as the most effective inhibitor across different *Phytophthora* life stages (Table 3.1-Table 3.4).

Table 3.1: The average effective concentration (µg/mL) of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin required to reduce *Phytophthora* spp. mycelial growth by 50% (EC_{50})
Hatched cells indicate that there was no information available regarding the fungicide for a particular *Phytophthora* spp.

	Effective concentration to reduce growth by 50% (EC_{50})				
Phytophthora spp.	Ethaboxam	Fluopicolide	Mandipropamid	Oxathiapiprolin	Reference
P. cinnamomi	0.035	0.046	0.133	0.0004	Belisle et al., 2019
P. infestans	0.052				Kim et al., 2004
		0.30	0.011		Saville et al., 2015
		0.44			Wang et al., 2014
				0.000511	Miao et al., 2016b
P. capsici	0.201				Kim et al., 2004
		0.204			Jackson et al., 2010
		0.23			Wu et al., 2020
		0.22			Keinath & Kousik, 2011
			0.075		Qi et al., 2012
				0.001	Ji & Csinos, 2015
				0.000678	Miao et al., 2016b
P. nicotianae	0.003	0.095	0.005	0.0006	Hao et al., 2019
				0.0044	Bittner & Mila, 2016
		0.09	0.04	0.001	Qu et al., 2016b
	0.016	0.057	0.005	0.0005	Gray et al., 2018
		0.020			Ren et al., 2018
P. citrophthora	0.17	0.041	0.004	0.0008	Hao et al., 2019
	0.068	0.04	0.004	0.0003	Gray et al., 2018
P. sojae				0.000321	Miao et al., 2016b
P. melonis				0.000477	Miao et al., 2016b
P. parasitica				0.000314	Miao et al., 2016b
P. syringae	0.005	0.045	0.003	0.0001	Gray et al., 2018
P. hibernalis	0.030	0.018	< 0.0003	≤ 0.001	Gray et al., 2018
P. litorale	0.266				Radmer et al., 2017
P. erythroseptica		0.18			Zhang et al., 2019

Table 3.2: The average effective concentration (µg/mL) of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin required to reduce *Phytophthora* spp. oospore production ≥ 90% ($EC_{≥90}$)

	Effective concentration to reduce growth by 50% (EC_{50})				
Phytophthora spp.	Ethaboxam	Fluopicolide	Mandipropamid	Oxathiapiprolin	Reference
P. nicotianae	0.1	0.4	0.02	0.0004	Gray et al., 2018

Table 3.3: The average effective concentration (µg/mL) of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin required to reduce *Phytophthora* spp. sporangial activity by 50% (EC_{50}) or ≥ 90% ($EC_{≥90}$)
Hatched cells indicate that there was no information available regarding the fungicide for a particular *Phytophthora* spp.

	Effective concentration to reduce growth by 50% (EC_{50})				
Phytophthora spp.	Ethaboxam	Fluopicolide	Mandipropamid	Oxathiapiprolin	Reference
P. infestans		≈3.9[1]			Wang et al., 2014
P. capsici		1.7[2]			Jackson et al., 2010
		0.105[2]			Wu et al., 2020
		0.048[2]			Keinath & Kousik, 2011
			0.004[1]		Qi et al., 2012
				0.0003[2]	Ji & Csinos, 2015
				0.0000129[2]	Miao et al., 2016b
P. nicotianae				0.0007[2]	Bittner & Mila, 2016
		0.15[2]	0.03[2]	0.0002[2]	Qu et al., 2016b
P. citrophthora	0.1*,[2]	0.28*,[2]	0.026*,[2]	0.005*,[2]	Gray et al., 2018

* Inhibition reported as $EC_{≥90}$ as opposed to EC_{50}

[1] Sporangia germination

[2] Sporangia production

Table 3.4: The average effective concentration (µg/mL) of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin required to reduce *Phytophthora* spp. zoospore activity by 50% (EC_{50}) or ≥ 90% ($EC_{≥90}$)
Hatched cells indicate that there was no information available regarding the fungicide for a particular *Phytophthora* spp.

	Effective concentration to reduce growth by 50% (EC_{50})				
Phytophthora spp.	Ethaboxam	Fluopicolide	Mandipropamid	Oxathiapiprolin	Reference
P. infestans		≈1.5[1]			Wang et al., 2014
P. capsici		2.7[2]			Jackson et al., 2010
		0.23[1]			Wu et al., 2020
		2.08[2], 0.10[3]			Keinath & Kousik, 2011
				0.54[2]	Ji & Csinos, 2015
				4.38[1]	Miao et al., 2016b
P. nicotianae				0.0041[2], 0.01[4]	Bittner & Mila, 2016
		0.16[2]	0.04[2]	0.002[2]	Qu et al., 2016b
P. citrophthora	>40[2]	>60[2]	0.026[2]	0.008[2]	Gray et al., 2018

* Inhibition reported as $EC_{≥90}$ as opposed to EC_{50}

[1] Zoospore release

[2] Zoospore germination

[3] Zoospore production

[4] Zoospore motility

3.1.3 Potential for a plant derived solution

A wide variety of indigenous Aotearoa plants contain bioactive compounds that display activity against microbes (Bloor, 1995; Calder et al., 1986). Kānuka and mānuka have been most widely studied for their pharmacological and antimicrobial properties, and both terpenes and triketones have been identified as the major sources of antimicrobial action (Costa et al., 2010; Lis-Balchin et al., 2000; van Klink et al., 2005).

With the persistent spread of kauri dieback, there has been additional interest in screening indigenous plants for anti-*Phytophthora* activity. This effort has focused on utilising mātauranga Māori, specifically with regard to identifying pioneer plants that are considered to both 'cleanse' and 'prepare' the soil for future generations of plants (Lawrence et al., 2019). While this is still an evolving area of research, plant extracts and soils from soft mingimingi (*Leucopogon fasciculatus*), kānuka, and mānuka as well as a range of natural products have been shown to inhibit certain life stages of *P. agathidicida* (Kentjens, 2019; Lawrence et al., 2019, 2021). In particular, three flavanone compounds have been isolated from kānuka leaves that inhibit both zoospore germination and mycelial growth of *P. agathidicida* (Lawrence et al., 2019). However, these flavanones are not yet commercially available and extraction of kānuka leaf tissue produces too low a yield for further *in vivo* testing to be currently feasible. In the meantime, it is worth considering the bioactive potential of plant essential oils and their major constituents, which are more readily available, as a possible means of *P. agathidicida* control.

Essential oils contain volatile compounds, which have been shown to inhibit *Phytophthora* mycelial growth *in vitro*, reduce *Phytophthora* populations in soils, and even suppress disease symptoms when applied directly to infected plant tissue (Bi et al., 2012). While some essential oils may inhibit seedling emergence or have other, negative physiological effects on plant growth, it is worth noting that these effects are usually less severe than the effect the disease itself (Bi et al., 2012).

There are commercially available essential oils for only two, indigenous Aotearoa plants — kānuka and mānuka. Kānuka essential oil has demonstrated antimicrobial properties (Lis-Balchin et al., 2000), however, it does not appear to have been screened against *Phytophthora* pathogens previously. It is also worth considered the bioactivity of essential oils from exotic plants, for which there are many candidates (Amini et al., 2016; Bi et al., 2012; Diánez et al., 2018; Lee et al., 2009; Wang et al., 2019), as *P. agathidicida* may be an exotic pathogen (Beever et al., 2010).

3.1.4 Proposed study

This study evaluated the effect of four fungicides and five essential oils on the viability of *P. agathidicida in vitro*. The fungicides that were tested are ethaboxam, fluopicolide, mandipropamid,

and oxathiapiprolin, which were screened against *P. agathidicida* mycelial growth, sporangia production, and oospore viability. The essential oils — kānuka, mānuka, tea tree (*Melaleuca alternifolia*), thyme (*Thymus vulgaris*), and rose geranium (*Pelargonium graveolens*) — were only tested on *P. agathidicida* mycelial growth, after which they were analysed by gas chromatography-mass spectrometry (GC-MS) to identify further, inhibitory compounds of interest.

3.2 Methods

3.2.1 Materials

Mandipropamid (32805) and fluopicolide (41132) were purchased as analytical standards from Merck. Ethaboxam (N-14143-50MG) and oxathiapiprolin (N-14266-10MG) were purchased as analytical standards from Chem Service, Inc. A sample of the fungicide Zorvec® Enicade® (active ingredient 100 g/L oxathiapiprolin) was provided by Corteva Agriscience. For the essential oils, kānuka, mānuka, and *P. graveolens* essential oil were purchased from PureNature while *M. alternifolia* and *T. vulgaris* essential oil were purchased from Go Native New Zealand.

3.2.2 Mycelial production

All three *P. agathidicida* isolates were routinely cultured on 20% cV8, the full methods for which are provided in Section 2.2.3.

3.2.3 Sporangia production

Fresh mycelial mats from *P. agathidicida* isolate NZFS 3770 were generated by incubating a 7 mm agar plug in a Petri dish with enough 10% carrot broth to just cover the plug. This particular growth media has been reported to lead to enhanced mycelial growth when compared to V8 broth (Armstrong, 2018). The Petri dishes were incubated at room temperature in the dark overnight, after which a dense mycelial fringe had grown around the plug.

Sporangia production is induced in *Phytophthora* spp. by incubating active mycelia in relatively nutrient depleted conditions under light (Drenth & Sendall, 2001). Thus, the carrot broth was decanted, and the mycelia was rinsed and incubated with an incubation media. The mycelial mats were washed and incubated with 10 mL incubation liquid three times. Each wash was incubated for 45 minutes at room temperature. After the last wash, a final 10 mL incubation liquid was added, and the Petri plates were incubated overnight under direct light at 22°C.

Incubation media for *Phytophthora* sporangia are typically soils extracts or pond water, both of which have been used successfully with *P. agathidicida* (Armstrong, 2018; Lawrence et al., 2017, 2019). While some previous *P. agathidicida* assays have used sterile incubation media (Lawrence et al., 2017, 2019),

other studies recommend non-sterile incubation media as autoclaved incubation liquids can reduce, or even prevent, sporangia production (Armstrong, 2018; Drenth & Sendall, 2001).

A variety of different non-sterile incubation media were trialled in this assay — pond water taken from a native planting conservation area in Halswell Quarry, Ōtautahi|Christchurch as well as 2% and 5% soil extracts prepared with soils take from podocarp forest in Riccarton Bush, Ōtautahi, pasture from a Lincoln University farm site, Waitaha|Canterbury, and healthy kauri from Titirangi, Tāmaki Makaurau|West Auckland. Pond water was filtered twice through paper filters (Whatman® Grade 1). A 10% (w/v) soil extract stock was prepared by adding 20 g soil to 200 mL sterile MQ^{+} and mixing with a magnetic stir bar for four hours, after which the soil settled out overnight. Soil extracts were decanted the following day, centrifuged at 3,220 x ***g*** for 20 minutes, filtered twice through paper filters (Whatman® Grade 1), and then further diluted to either 2% or 5% using sterile MQ^{+}. The effect of incubation media on sporangia formation was conducted using biological triplicates.

Mycelia was observed after 16 hours, however, unlike other studies (Armstrong, 2018; Lawrence et al., 2017, 2019), mature sporangia were not observed until after 36 hours of incubation under light. Sporangia were visualised using lactophenol cotton blue and acid fuchsin, the latter of which is commonly used to visualise *Phytophthora* sporangia (Jackson et al., 2010; Ji & Csinos, 2015; Qu et al., 2016b; Weir et al., 2015). Sporangia were viewed using an Olympus BX41 microscope at 40x magnification. Sporangial counts were taken using four microscope fields for all mycelial mats. Additionally, for a subset of samples, sporangia counts were conducted for the whole mycelial mat.

3.2.4 Oospore production

Phytophthora agathidicida oospores were produced and isolated according to the methods previously described in Sections 2.2.6 and 2.2.8 respectively.

3.2.5 Dissolution assay

Essential oils contain both volatile and lipophilic compounds (Turek & Stintzing, 2013), the latter of which make essential oils immiscible in water. Prior to screening the essential oils for anti-*Phytophthora* activity, they were mixed with a range of solvents and surfactants based on materials used in previous studies (Amini et al., 2016; Bi et al., 2012; Diánez et al., 2018; Kotan et al., 2013; Othman et al., 2017; Sarkhosh et al., 2018; Wang et al., 2019).

All dissolution trials were conducted by preparing a 1 mL aliquot of 5% essential oil in RO water, 2%, 10%, or 100% dimethyl sulfoxide (DMSO), 96% ethanol, or 100% acetone that had been either amended with 0.01% Tween 20, 0.05% or 0.5% Tween 80, or non-amended. Each aliquot was vortexed briefly to mix, after which the solution settled for a period of five minutes. Aliquots were evaluated

based on whether the oil had dissolved completely, formed an emulsion layer, or if the oil had completely separated out from the solution.

As it has been well documented that ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin readily dissolve in acetone (Belisle et al., 2019; Gray et al., 2018; Hao et al., 2019), they were not subjected to a dissolution assay.

3.2.6 Disc diffusion assay

Potential anti-*Phytophthora* compounds were assessed using a modified Kirby-Bauer disc diffusion assay (Bauer et al., 1966). This assay often serves as a precursor to more rigorous methods due to its simplicity, low cost, and high-throughput screening capacity (Balouiri et al., 2016; Edelmann et al., 2007).

As per previous disc diffusion assays with *P. agathidicida*, four concentrations of each fungicide or essential oil were prepared (Lawrence et al., 2017). Ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin were dissolved in acetone to concentrations of 0.05, 0.5, 5, and 50 µg/mL. The essential oils were dissolved in acetone to concentrations of 0.01, 0.1, 1, and 5 µL/mL. If no inhibition zone was produced using these concentrations, a second screening was conducted using concentrations of 75, 150, 300, and 500 µg/mL for the fungicides or 10, 50, 250, and 500 µL/mL for the essential oils.

Sterile, 6 mm paper discs (Whatman® Grade 1) were imbued with 5 µL of either diluted, inhibitory solution or acetone as a control, after which they were dried under sterile conditions at room temperature for 30 minutes. The disc diffusion assay was set up by placing a 5 mm agar plug of active *P. agathidicida* mycelia in the centre of a 90 mm Petri plate containing 20% cV8 agar. The bottom of the Petri plate was inscribed with a 70 mm circle. For the fungicides, four discs containing dilute fungicide of different concentrations along with an acetone control disc were spaced evenly around the inscribed circle (Figure 3.1a). As the volatile compounds in essential oils have been shown to act as a fumigant to disrupt mycelial growth of phytopathogenic fungi (Bi et al., 2012; Lee et al., 2009), multiple concentrations of dilute essential oil were not tested on the same plate. Instead, a single concentration was placed 180° from an acetone control disc (Figure 3.1b). Three biological replicates of *P. agathidicida* isolate NZFS 3770 were used for each fungicide and essential oil screened. Full control plates were also established, in which all paper discs were imbued with acetone.

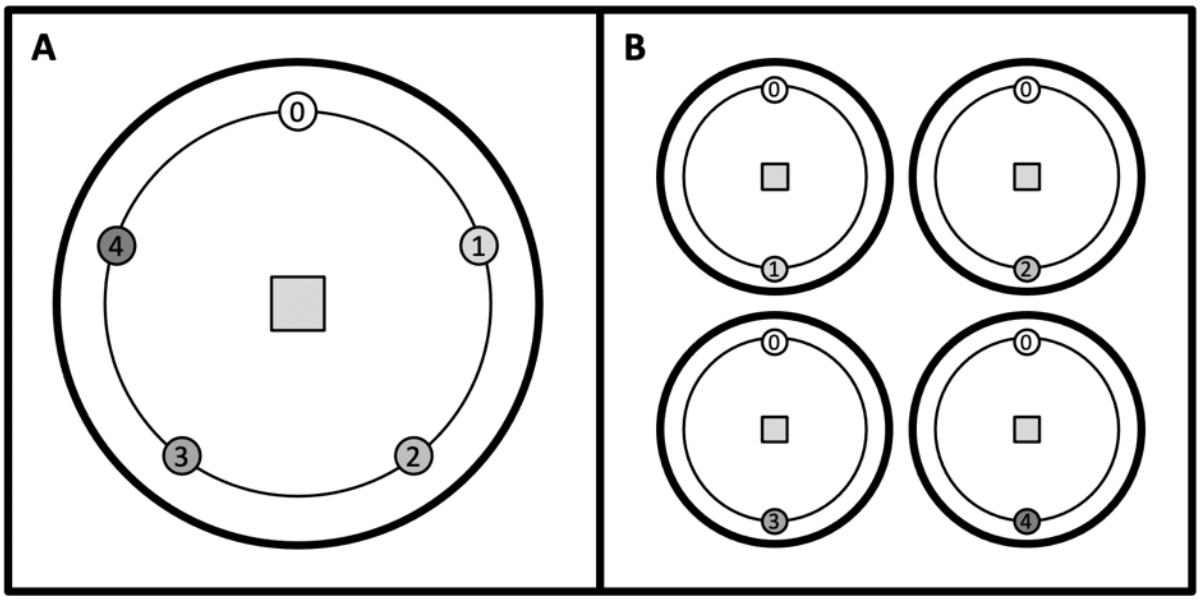

Figure 3.1: High-throughput disc diffusion assay set up for anti-*Phytophthora* candidates
a| Set up for fungicides, and b| essential oils. The discs placed along the inscribed circle were imbued with acetone (labelled as 0) or varying concentrations of dilute fungicide or essential oil, with concentrations increasing from 1 to 4. Figure produced by Thurston, (2021).

All plates were sealed with Parafilm and incubated in the dark at 22°C for six days, at which point the control plates had grown to the edge of the inscribed circle. Mycelial growth was measured from the edge of the agar plug to the edge of visible hyphae using a ruler to one decimal place. Radial growth for each concentration was compared to that of an acetone control to assess for inhibition potential.

3.2.7 Mycelial growth EC_{50} assay

The agar dilution method involves amending molten agar with different concentrations of an antimicrobial candidate, typically using a series of two-fold dilutions, which is then poured and inoculated with a standard amount of inoculum (Balouiri et al., 2016). This method is commonly used to determine the MIC or EC_{50} value of antimicrobial candidates (Bonev et al., 2008).

Acetone is toxic to microorganisms at certain concentrations (Dyrda et al., 2019), primarily through its ability to affect cell membrane fluidity (Ingram, 1977). As acetone was to be the carrier for all potential anti-*Phytophthora* candidates, it was necessary to determine its inhibitory effect on the mycelial growth of *P. agathidicida*. To do so, acetone was added to 10 mL aliquots of molten 20% cV8 agar to yield a final concentration of 0, 0.25, 0.5, 1, 2, or 5%. The molten agar was swirled vigorously to mix and then immediately poured into a 60 mm Petri dish. Plates set under UV light for one hour, after which they were inoculated with a 3.8 mm agar plug from 20% cV8 agar containing *P. agathidicida* isolate ICMP 18970 active mycelia. Three biological replicates were used for each acetone concentration. After four days, the diameter of the mycelial growth was measured at four, equidistant, points using digital callipers (ROK DC-162MA) to two decimal places. Mycelial inhibition was calculated

using Equation 1 below, where C_d refers to the average diameter of the control and T_d refers to the average diameter of the treatment:

Equation 1.

$$\textbf{Inhibition} = (C_d - T_d)/\ C_d \times 100$$

After determining the inhibition potential of acetone, molten agar was amended with 50 µL of an anti-*Phytophthora* candidate diluted in acetone for a final concentration of 0.5% acetone in the growth media. These agar dilution assays were conducted by using eight, two-fold dilutions of each fungicides and essential oil. The concentrations used for the fungicides ranged from 1.56×10^{-5} to 4 µg/mL whereas the concentrations used for essential oils ranged from 4.88×10^{-3} to 50 µL/mL. For the fungicides, this assay was conducted using three biological replicates for each of the three *P. agathidicida* isolates. For the essential oils, this assay was conducted using three biological replicates of isolate ICMP 18970.

EC_{50} values were estimated by log-transforming the concentration and fitting the data with a non-linear regression using GraphPad Prism version 6.0 (Lawrence et al., 2017).

3.2.8 Chemical composition of essential oils

Following the methods described by Diánez et al. (2018), 1 mL essential oil was diluted in 10 mL 99.8% HPLC grade dichloromethane (402152, Thermo Fisher Scientific) and mixed thoroughly. A subsample of each dilution was transferred to a 2 mL vial for GC-MS analysis.

Volatile organic compounds present in each samples were analysed based on the methods outlined by Hudaib et al. (2002). Briefly, 0.5 µL of each essential oil was injected into a Shimadzu QP2010 GC-MS (Shimadzu Scientific Instruments, Oceania, NSW, Australia) with an injector operating in split mode (100:1) at 250 °C with a constant helium flow rate of 1 mL/min (linear velocity of 36.2 cm/sec). An Rtx-5ms capillary column (30 m x 0.25 mm id x 0.25 µm film thickness, 5% phenyl 95% dimethyl polysiloxane, Restek , Bellefonte, PA, USA) was used to separate the volatile organic compounds using an oven ramp with an initial temperature of 45°C held for 10 minutes, then ramped to 180°C at 2.5 °C/min and held for a further 5 minutes before a final ramp to 250 °C at 30 °C/min with a final hold time of 5 minutes giving a total run time of 76.33 minutes (Hudaib et al., 2002). The mass spectral detector (EI, 70 eV) was operated in scan mode at a range of 40 – 400 m/z (Diánez et al., 2018) with the total ion current (TIC) recorded for all chromatograms. The temperature of the transfer line and ion source were maintained at 200°C and 250°C respectively.

Compounds were identified by comparing their mass spectral patterns with those in mass spectral libraries NIST 11 (National Institute of Standards and Technology, 2012) and Wiley 10 (John Wiley & Sons, 2013) and validated by confirming the published retention indices (Boukhatem et al., 2013; Costa et al., 2010; da Silva et al., 2012; Ghannadi et al., 2012; Hudaib et al., 2002; Khan et al., 2019; Park et al., 2017), which were calculated based on running an alkane mix C_8-C_{20} (Sigma Aldrich, New Zealand) under the same chromatographic conditions as previous studies (van Den Dool & Kratz, 1963; Zellner et al., 2008). The quantitative concentrations (as % content) were calculated by comparing the peak area (TIC) of each compound with the total peak area of all detected peaks (TIC).

3.2.9 Sporangia production EC_{50} assay

Fresh mycelial was generated by incubating a 7 mm agar plug containing active *P. agathidicida* mycelia in 1 mL 10% sterile carrot broth in a 12-well tissue culture plate (TCP-000-012, BIOFIL®). Plates were incubated overnight, after which the broth was removed via pipette and the mycelia was washed and incubated three times with non-sterile pond water that had been amended with six concentrations of each fungicide based on the results from the mycelial inhibition assay (See Section 3.3.4). Three biological replicates of *P. agathidicida* isolate NZFS 3770 were used for each fungicide and concentration. The washes were incubated at room temperature for 45 minutes, after which the mycelial plugs were incubated with 1 mL amended pond water for 36 hours. Sporangia, both intact and empty (Figure 3.2), were counted for four microscope fields at 40x magnification using an Olympus BX41 microscope.

3.2.10 Oospore viability EC_{50} assay

Due to time constraints, not all fungicides nor *P. agathidicida* isolates could be used in oospore inhibition trials. As oxathiapiprolin produced the greatest inhibitory effect in the mycelial trial (Section 3.3.4), it was chosen as the sole fungicide to be used against *P. agathidicida* isolate NZFS 3770.

Isolated oospores were incubated in the dark at room temperature with oxathiapiprolin at four, 10x dilutions ranging from 7.81×10^{-6} to 7.81×10^{-3}. Subsamples from each treatment were removed after 2, 4, 12, 24, and 48 hours, which were then diluted at a 1:1 ratio with 0.1% MTT in sterile MQ^+ at 35°C for 48 hours. For each sample, 100 spores were observed and photographed at 40x using an Olympus DP74 microscope digital camera. Spores were evaluated by assigning the stain classifications outlined in Figure 2.12.

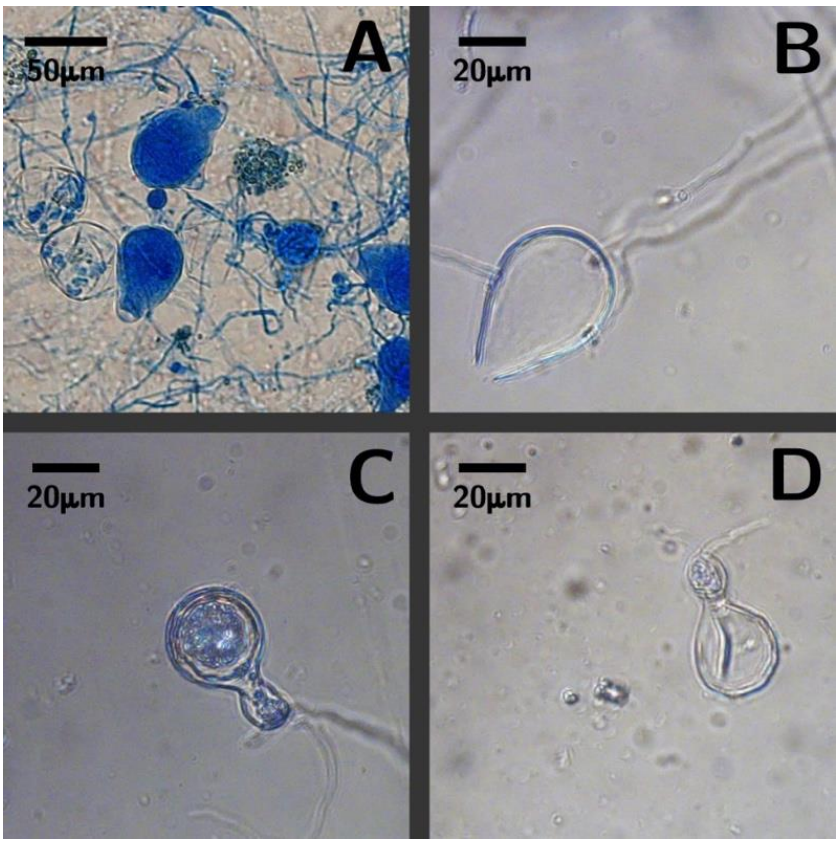

Figure 3.2: Appearance of spores generated by incubating *Phytophthora agathidicida* mycelial with nutrient-poor media
a| Blue-stained mature sporangia that have not yet released zoospores. b| Empty sporangia after discharging sporangia. c| A mature, thick-walled oospore with an intact ooplast. d| An aborted oospore, devoid of ooplast.

3.3 Results

3.3.1 Dissolution assay

As expected, none of the essential oils dissolved in RO water. Neither kānuka nor mānuka essential oil dissolved in DMSO of any concentration, and the remaining essential oils did not dissolve in 2% or 10% DMSO.

Essential oils that were mixed with solutions of Tween 20 and 80 in either RO water or DMSO yielded an emulsion layer. The vast majority of these were characterised as an unstable emulsion layer, as the layer was situated on the surface of the solution and progressively separated out over time.

All essential oils dissolved in 96% ethanol or 100% acetone. As the fungicides were to be dissolved and diluted in acetone, acetone was also chosen to be the dissolution solvent for the essential oils.

3.3.2 Disc diffusion assay

All fungicides and essential oils produced a zone of inhibition (Figure 3.3, Table 3.5), albeit at concentrations higher than what was expected based on EC_{50} values for *Phytophthora* spp. mycelial inhibition in previous studies (Table 3.1).

Based on the unexpectedly high MIC approximated by this assay, all four initial concentrations of both fungicides and essential oils were re-tested in a preliminary agar dilution assay, after which a more appropriate concentration range was selected.

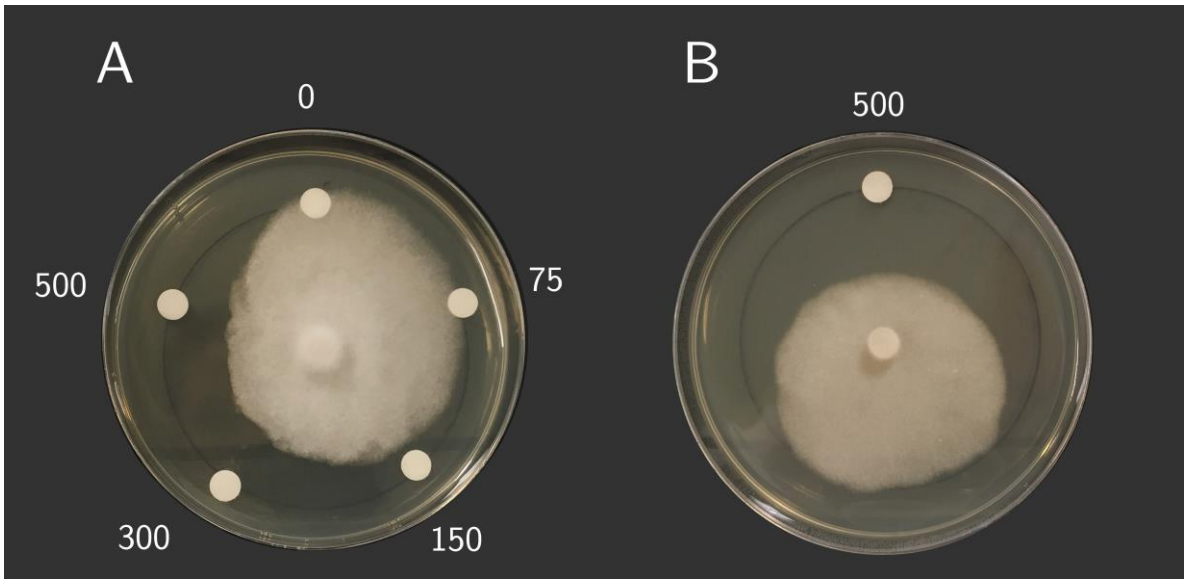

Figure 3.3: Mycelial growth of *Phytophthora agathidicida* in the presence of inhibitors in a disc diffusion assay
a| Fluopicolide. b| *Thymus vulgaris* essential oil. The concentration imbued on each paper disc is reported in µg/mL for fluopicolide and µL/mL for *T. vulgaris* essential oil.

3.3.3 Acetone inhibition assay

Increasing the concentration of acetone in the growth media was positively correlated (R^2 =0.997) to increasing inhibition of mycelial growth (Figure B.1). Using a concentration of 0.25% acetone had a negligible effect on mycelial growth (1.41 ± 1.41% STE), however, it was determined that amending the agar media at this concentration was not feasible. This was due to both the high costs and low quantities of the fungicides used in this experiment, which could not be concentrated to the degree required using 0.25% acetone. Thus, the media used in the following mycelial growth EC_{50} assay had a final concentration of 0.5% acetone, which inhibited mycelia growth by 7.77 ± 1.81% STE.

3.3.4 Mycelial growth EC_{50} assay

The MICs necessary to inhibit *P. agathidicida* mycelial growth as determined by the agar dilution assay were considerably lower than anticipated given the results of the disc diffusion assay (Table 3.5**,** Figure 3.4), generally by two orders of magnitude.

Oxathiapiprolin most effectively inhibited *P. agathidicida* growth across the three isolates studied (Figure 3.6). The EC_{50} value for oxathiapiprolin estimated by this study agrees with that which was recently released as a pre-print (Lacey et al., 2021a), 2.0×10^{-4} and 1.0×10^{-4} µg/mL respectively. The commercial formulation Zorvec® Enicade® was approximately two times less effective than the

analytical standard, however, even this was about two orders of magnitude more effective than mandipropamid, the next most potent fungicide. Out of all the fungicides tested, fluopicolide had the highest average EC_{50} value (≈3.69 × 10^{-1}), although it was still considerably more effective than the essential oils. For fluopicolide and oxathiapiprolin, there were distinct morphology changes that occurred with increasing fungicide concentration (Figure 3.5).

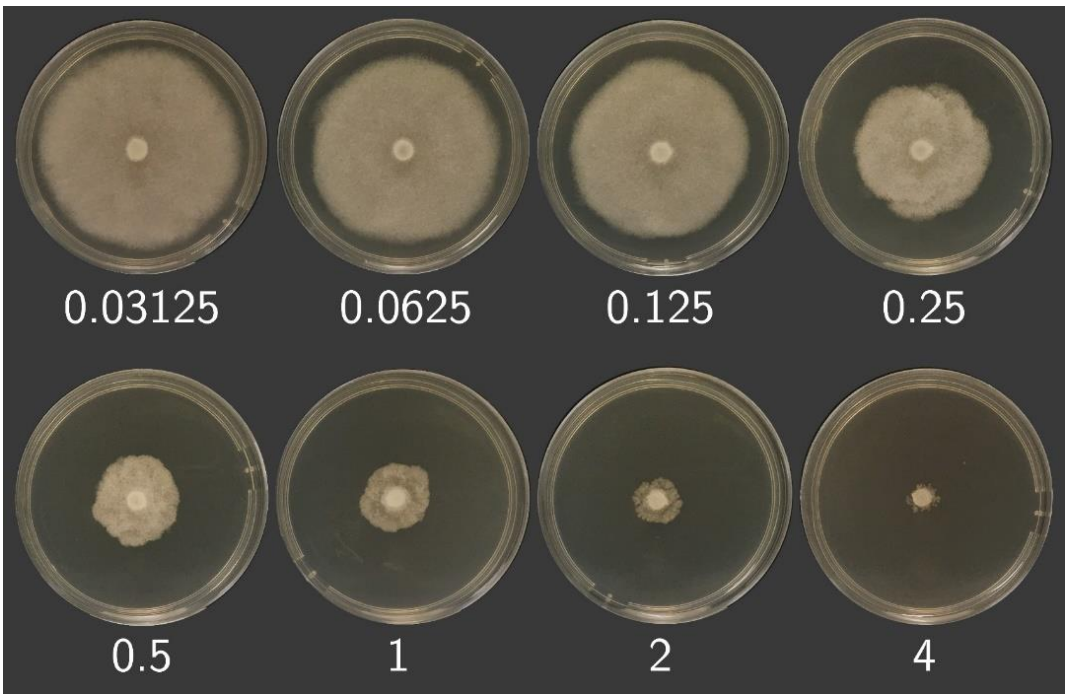

Figure 3.4: Mycelial growth of *Phytophthora agathidicida* in the presence of fluopicolide in an agar dilution assay
The concentration (µg/mL) of fungicide amended to the agar is listed below each plate.

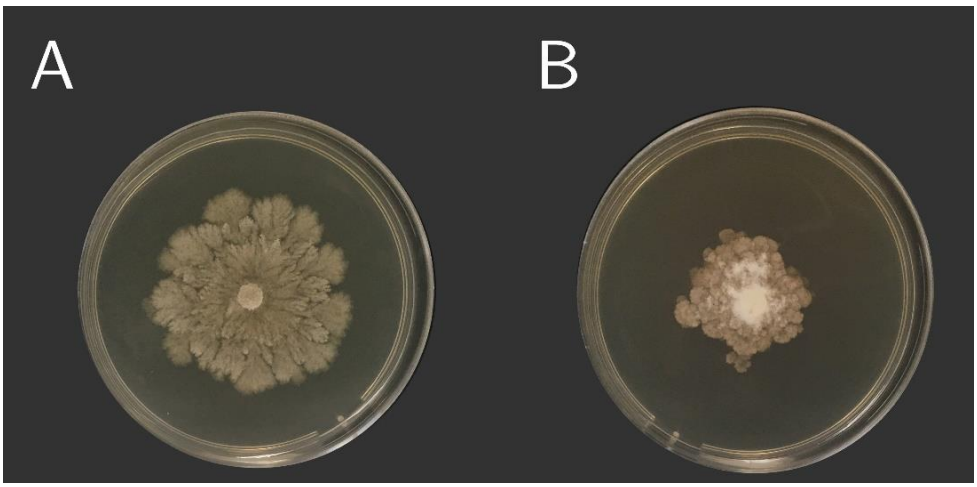

Figure 3.5: Changes in *Phytophthora agathidicida* morphology using fungicides
A| Fluopicolide and B| oxathiapiprolin.

While increasing concentrations of mānuka essential oil inhibited *P. agathidicida* mycelial growth, the inhibition observed was too low to calculate an EC_{50} value (Figure 3.7). Kānuka essential oil did not produce a typical inhibition curve, however, there was high enough inhibition observed to estimate an

EC_{50} value. *P. graveolens* and *T. vulgaris* essential oil most effectively inhibited *P. agathidicida* mycelial growth, with EC_{50} values of 2.11×10^{-1} and 7.88×10^{-2} µL/mL respectively.

Table 3.5. Inhibition of *Phytophthora agathidicida* (isolates NZFS 3770, ICMP 18969, and ICMP 18970) mycelia using fungicides and essential oils (EOs)
Minimum inhibitory concentration (MIC) was reported based on the lowest concentration that produced inhibition as determined by the disc diffusion assay, whereas the half maximal effective concentration (EC_{50}) was approximated from the agar dilution assay. The MIC and EC_{50} values were reported in µg/mL for fungicides and µL/mL for essential oils. The range in parenthesis is the 95% confidence interval.

	MIC (µg/mL or µL/mL)	EC_{50} (µg/mL or µL/mL)		
	NZFS 3770	NZFS 3770	ICMP 18969	ICMP 18970
Ethaboxam	50	7.24×10^{-2} (6.71×10^{-2}—7.80×10^{-2})	8.32×10^{-2} (7.62×10^{-2}—9.06×10^{-2})	1.04×10^{-1} (9.41×10^{-2}—1.14×10^{-1})
Fluopicolide	150	3.03×10^{-1} (2.55×10^{-1}—3.66×10^{-1})	4.14×10^{-1} (3.45×10^{-1}—5.16×10^{-1})	3.89×10^{-1} (3.35×10^{-1}—4.59×10^{-1})
Mandipropamid	5	2.12×10^{-2} (1.99×10^{-2}—2.24×10^{-2})	1.83×10^{-2} (1.63×10^{-2}—2.05×10^{-2})	2.22×10^{-2} (2.12×10^{-2}—2.34×10^{-2})
Oxathiapiprolin	Not tested	1.30×10^{-4} (1.25×10^{-4}—1.36×10^{-4})	1.65×10^{-4} (1.52×10^{-4}—1.79×10^{-4})	1.70×10^{-4} (1.59×10^{-4}—1.81×10^{-4})
Zorvec® Enicade®	1×10^{-3}	3.31×10^{-4} (2.99×10^{-4}—3.69×10^{-4})	3.96×10^{-4} (3.63×10^{-4}—4.36×10^{-4})	3.06×10^{-4} (2.75×10^{-4}—3.48×10^{-4})
Mānuka EO	50	Not tested	Not tested	Not detected
Kānuka EO	Not detected	Not tested	Not tested	3.28 (2.96—3.64)
Tea Tree EO	50	Not tested	Not tested	1.19 (1.14—1.24)
Rose Geranium EO	50	Not tested	Not tested	2.11×10^{-1} (1.89×10^{-1}—2.36×10^{-1})
Thyme EO	250	Not tested	Not tested	7.88×10^{-2} (7.49×10^{-2}—8.27×10^{-2})

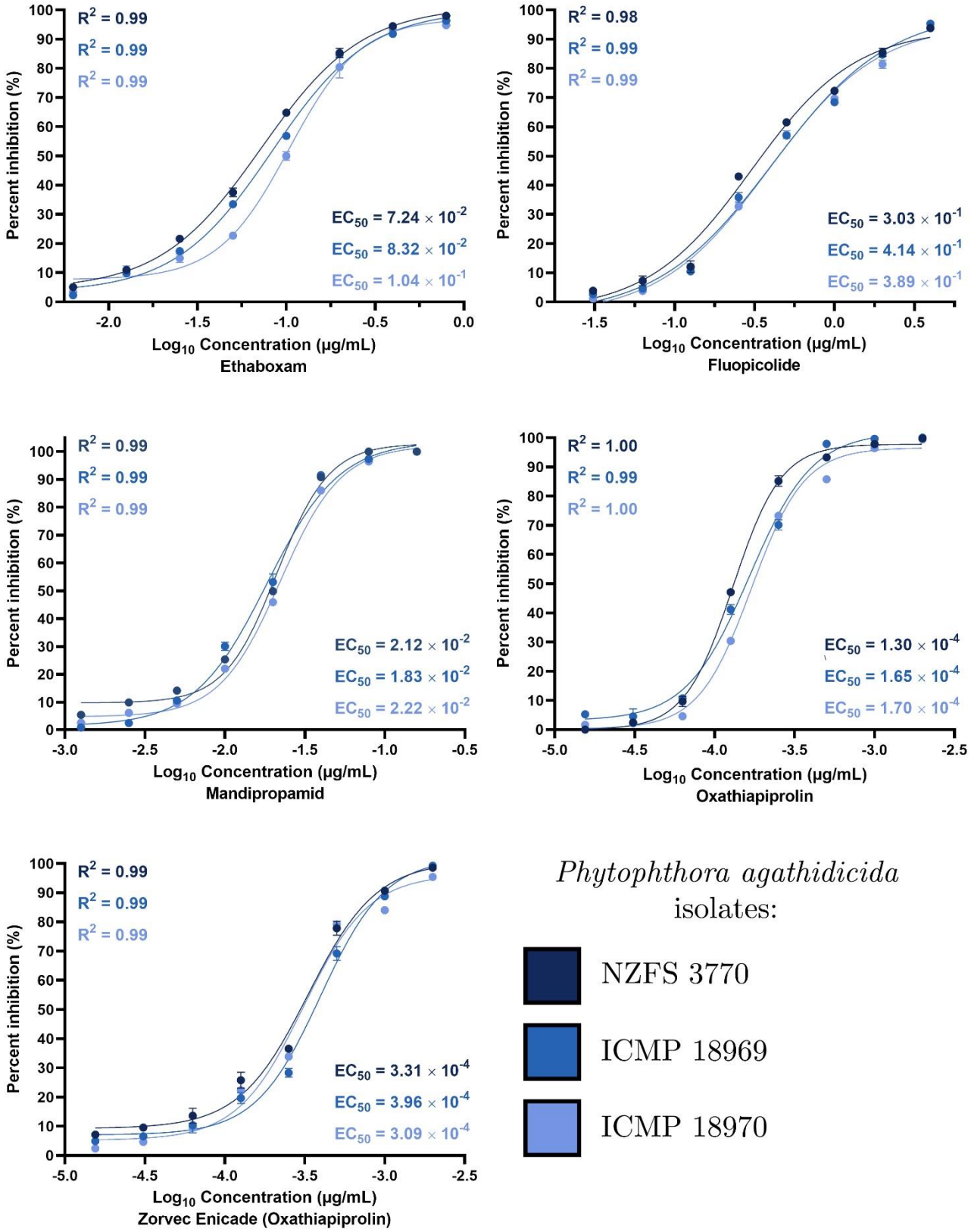

Figure 3.6: *Phytophthora agathidicida* (isolates NZFS 3770, ICMP 18969, and ICMP 18970) mycelial growth inhibition curves using anti-oomycete fungicides

Each line colour represents one of the three *P. agathidicida* isolates studies. The error bars depict standard error based on three, biological replicates.

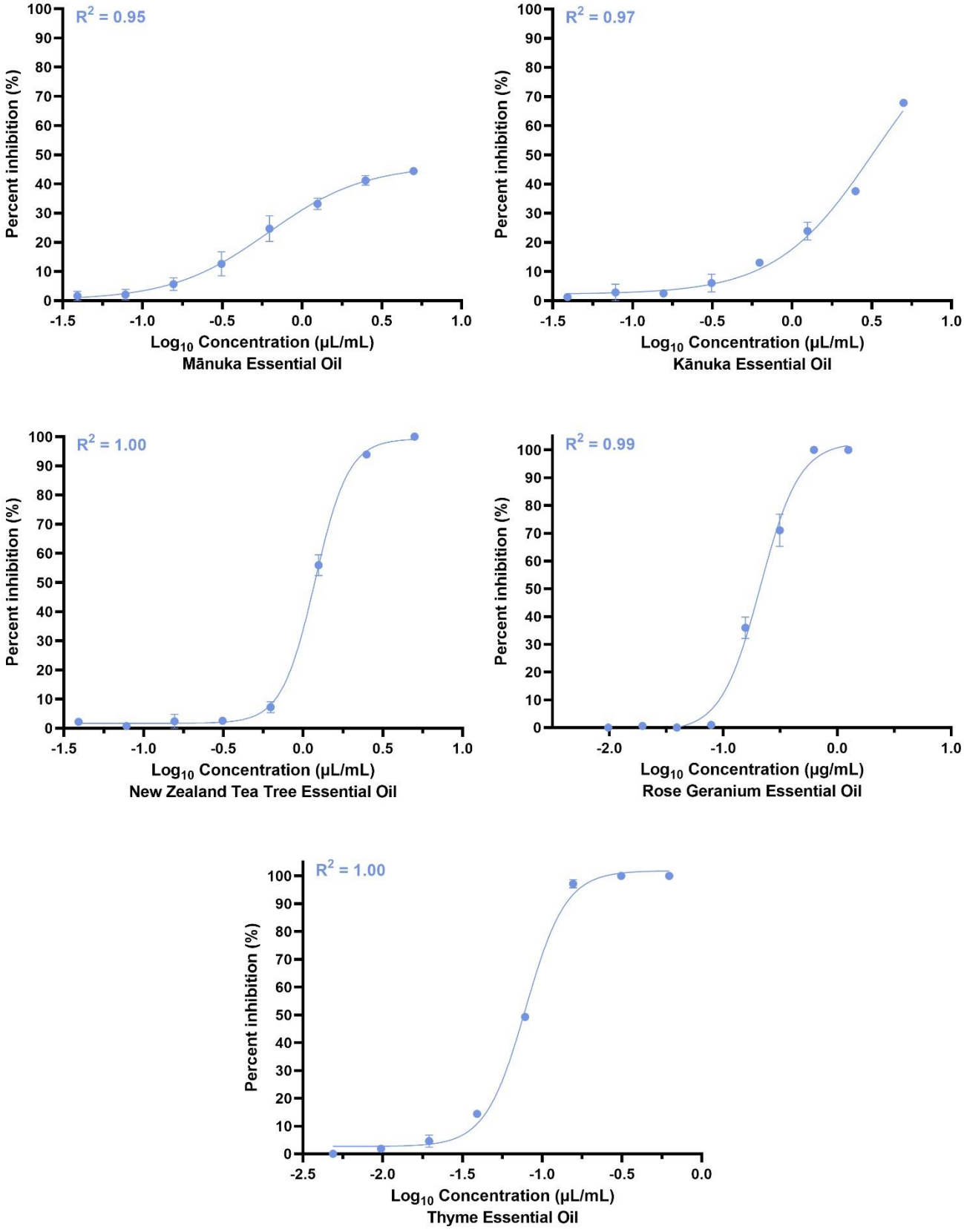

Figure 3.7: *Phytophthora agathidicida* isolate ICMP 18970 (isolated from Waipoua, Te Tai Tokerau|Northland in 2011) mycelial growth inhibition curves using five essential oils
The error bars depict standard error based on three, biological replicates.

3.3.5 Compounds identified by GC-MS

The major chemical compounds identified in the five essential oils are shown in Table 3.6. Copies of the raw GC-MS results can be found in Table B.1-Table B.5. Most compounds identified across all essential oils can be classified as terpenes — polyphenolic terpenes, monoterpenes, monoterpenoids, sesquiterpenes, and sesquiterpenoids.

Kānuka and mānuka essential oils had the most unidentified compounds. In particular, flavonoids and certain β-triketones, which have been well documented in extracts from these plants, were not found in these essential oils (Lawrence et al., 2019; Park et al., 2017). In some cases, this suggests that the GC-MS library does not have these compounds registered. For example, neither isoleptospermone nor leptospermone was identified using the GC-MS library and had to be resolved using previously published retention index (RI) values. Alternatively, some notable bioactive compounds present in plant exacts may not have been present in the essential oils, perhaps as a consequence of the quality of essential oil used. For example, there was no probable peak for flavesone in either kānuka or mānuka, which has been previously reported as a major essential oil constituent (Park et al., 2017), and no peaks were observed in kānuka essential oil in the range in which the three anti-*Phytophthora* flavanones are meant to appear (Lawrence et al., 2019).

3.3.6 Generation of *P. agathidicida* sporangia

Non-autoclaved, filtered pond water from Halswell Quarry, Ōtautahi generated the highest number of *P. agathidicida* sporangia (Figure 3.8). Apart from soil extracts produced from pine forest soil, a greater number of sporangia were produced using more highly concentrated soil extracts. Additionally, sporangia were produced at higher rates using soil extracts from landscapes dominated by indigenous endemic plants (e.g., kauri forest in Titirangi, Tāmaki Makaurau and the Riccarton Bush, Ōtautahi) when compared to landscapes that have been converted to pasture and exotic pine forests.

This dataset was significantly skewed and could not be normalised. Thus, no additional statistics have been performed on it and filtered pond water was chosen as the incubation media for additional sporangia assays.

Table 3.6: GC-MS identification, retention index (RI), and percent peak area for five essential oils.

Compound	RI Values[1]	*T. vulgaris*	*M. alternifolia*	*P. graveolens*	Mānuka	Kānuka
α-Thujene	921	0.07%	0.62%			0.59%
α-Pinene	925	0.57%	2.29%	0.46%	1.09%	54.05%
Camphene	938	0.05%				0.11%
β-Pinene	968	2.07%	1.16%		0.17%	0.53%
β-Myrcene	991	0.40%	0.80%	0.12%	0.19%	0.06%
3-Carene	1001		0.36%			
α-Carene	1014		8.31%			
α-Phellandrene	1022		1.59%	0.08%		6.91%
***p*-Cymene**	1023	22.66%				
Limonene	1026	0.52%	1.88%		0.08%	0.98%
Eucalyptol	1028		13.72%		0.13%	7.54%
γ-Terpinene	1059	30.28%	16.23%		0.10%	0.26%
α-Terpinolene	1085		2.96%			0.12%
Terpinoline	1087	0.08%				0.27%
Linalool	1088			0.14%		
α-Pinene oxide	1096		0.28%			
trans-Rose oxide	1101			4.37%		
cis-Rose oxide	1110			1.16%		
p-Menth-2-en-1-ol	1119		0.25%			
p-Menth-2-en-1-ol trans	1139		0.17%			
Menthone	1150			2.71%		
Isomenthone	1160			4.61%		
Borneol	1167	0.87%				
Terpinen-4-ol	~1175	0.18%	33.57%			
α-Terpineol	~1190	0.10%	3.41%	0.34%		1.65%
Citronellol	1236			32.45%		
Neral	1242			1.04%		
Geraniol	1261			13.91%		
Geranial	1273			0.67%		
Citronellyl formate	1278			7.67%		
Thymol	1303	39.37%				
Geranyl formate	1304			2.79%		
α-Cubebene	1345		0.06%	0.24%	3.52%	0.32%
Citronellyl acetate	1357			0.36%		
Isoledene	1370		0.11%			
α-Copaene	1370			0.60%	4.62%	0.87%
β-Bourbonene	1378			1.28%		
α-Gurjunene	1411		0.55%		3.47%	0.23%
γ-Caryophyllene	1411			1.28%		
Caryophyllene	1421		0.06%			
Alloaromadendrene	1431		1.21%			0.64%
trans-Muurola-3,5-diene	1445				5.12%	0.15%
Citronellyl propionate	1477			1.05%		
γ-Muurolene	1480				7.64%	

Continued on next page

Compound	RI Values[1]	*T. vulgaris*	*M. alternifolia*	*P. graveolens*	Mānuka	Kānuka
Cadina-1.4-diene	1485		0.18%			
Ledene	1490		1.92%			
Germacrene D	1490			0.74%		
α-Selinene	~1495			0.33%	7.74%	0.22%
Citronellyl butyrate	1516			0.08%		
δ-Cadinene	1520		1.73%	1.42%		
γ-Cadinene	1522				22.57%	4.57%
Calamenene	1528			0.10%	5.83%	0.21%
Geranyl butyrate	1563			0.88%		
β-Phenylethyl tiglate	1585			0.92%		
Geranyl isovalerate	1604			0.10%		
10-epi-γ-Eudesmol	1613			4.47%		
Isoleptospermone	1618				1.34%	0.09%
Geranyl valerate	1626			0.22%		
Leptospermone	1627				2.76%	0.21%
γ-Eudesmol	1644			0.28%		
Citronellyl tiglate	1668			0.30%		
Geranyl tiglate	1703			1.08%		
Sum		97.20%	93.42%	88.23%	66.37%	80.59%

[1] Retention index values based on a non-polar column (HP-5MS).

3.3.7 Generation of *P. agathidicida* sporangia

Non-autoclaved, filtered pond water from Halswell Quarry, Ōtautahi generated the highest number of *P. agathidicida* sporangia (Figure 3.8). Apart from soil extracts produced from pine forest soil, a greater number of sporangia were produced using more highly concentrated soil extracts. Additionally, sporangia were produced at higher rates using soil extracts from landscapes dominated by indigenous endemic plants (e.g., kauri forest in Titirangi, Tāmaki Makaurau and the Riccarton Bush, Ōtautahi) when compared to landscapes that have been converted to pasture and exotic pine forests.

This dataset was significantly skewed and could not be normalised. Thus, no additional statistics have been performed on it and filtered pond water was chosen as the incubation media for additional sporangia assays.

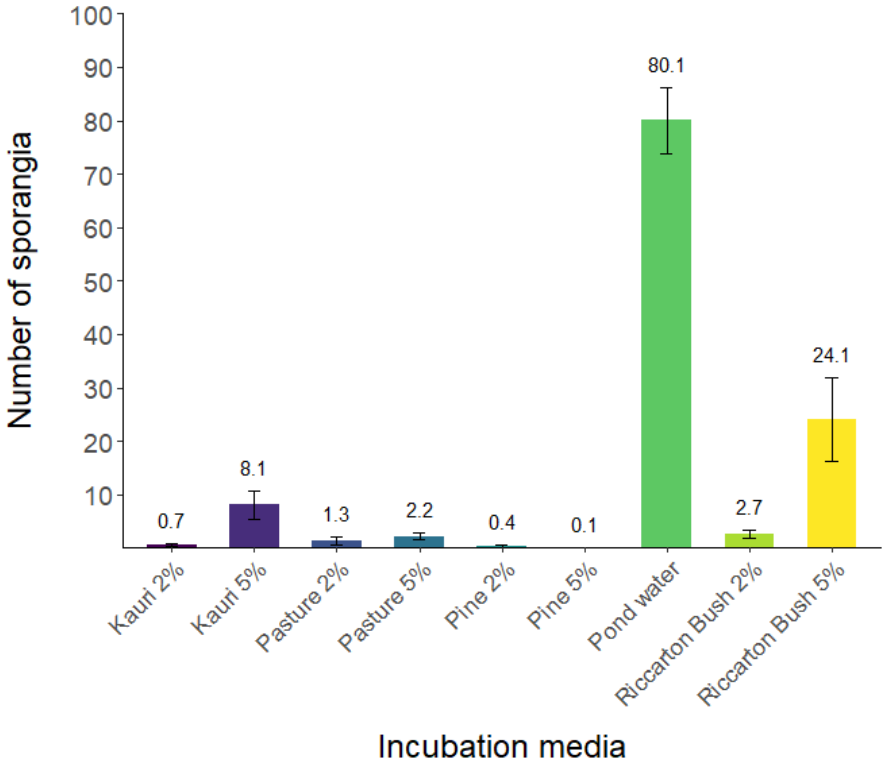

Figure 3.8: Generation of *Phytophthora agathidicida* isolate NZFS 3770 sporangia using low-nutrient incubation liquids
The error bars depict standard error and the number above each bar describes the average number of sporangia produced based on three replicates.

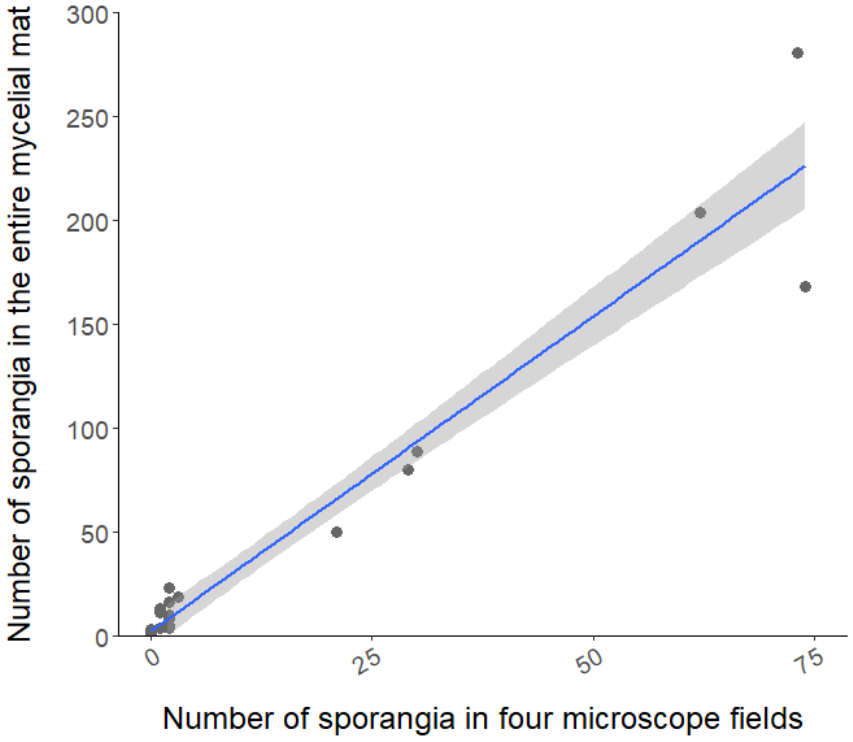

Figure 3.9. Correlation between *Phytophthora agathidicida* sporangia count data using either four microscopy fields or the entire mycelial mat
The data has been fit with a linear regression (R^2=0.94) and the grey region is the 95% confidence interval.

3.3.8 Sporangia production EC_{50} assay

There was high correlation (R^2 = 0.94) between sporangia counts using the two methods applied (Figure 3.9). Thus, in all subsequent studies, sporangia were counted in four microscope fields, which significantly reduced sample processing time.

Both ethaboxam and fluopicolide inhibited sporangia production, with EC_{50} values of 7.44×10^{-3} µg/mL (95% C.I. 4.08×10^{-3} — 9.77×10^{-3}) and 4.10×10^{-1} µg/mL (95% C.I. 2.44×10^{-1} — 7.63) respectively (Figure 3.10). Interestingly, the EC_{50} value for inhibition of sporangia production using ethaboxam was lower than that reported for mycelial growth inhibition (Table 3.5), which could indicate that this life cycle stage is more sensitive to ethaboxam. The EC_{50} value for fluopicolide was similar across both inhibition assays, however, the fit of the data, and consequently the 95% confidence interval, was much worse for the sporangia production assay. This could be attributed to the fact that there were fewer concentrations tested but is more likely due to the high variation in sporangia production between biological replicates.

In general, sporangia production was more variable in comparison to mycelia production. For both mandipropamid and oxathiapiprolin, EC_{50} values could not be calculated due to abnormally low sporangia production in controls coupled with no clear data trend with increasing fungicide concentrations. Further assays were conducted to try and elucidate EC_{50} values for mandipropamid and oxathiapiprolin as well as test a lower concentration range for ethaboxam. However, sporangia production continued to be inconsistent, particularly in controls, and thus, no further data could be collected.

3.3.9 Oospore viability EC_{50} assay

There was no difference in oospore activity (e.g., dormant or active) or overall viability across the 48-hour sampling period at any concentration tested. Interesting, blue stained oospores accounted for 5.5 ± 1.2% of all spores (Figure 3.11), which had not been observed in previous MTT viability studies conducted during this research (Section 2.3.3).

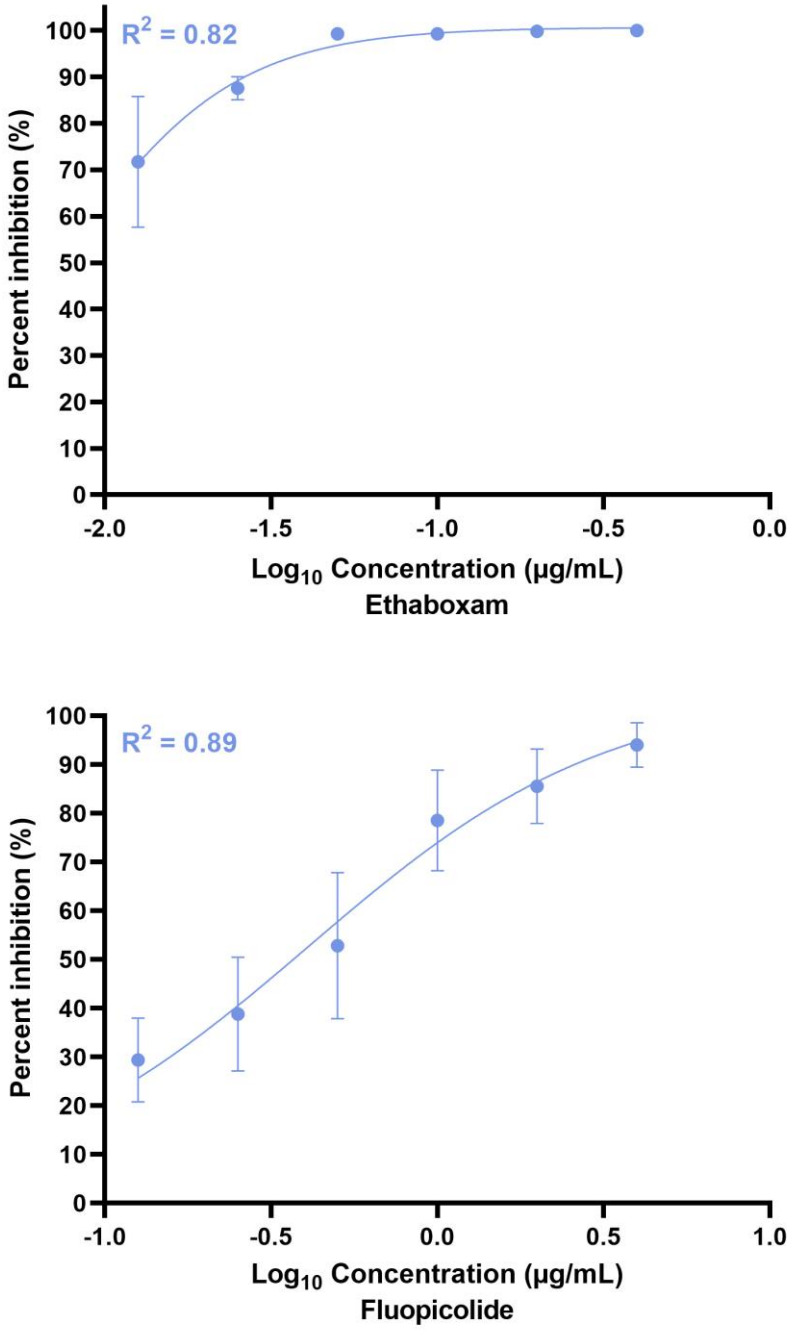

Figure 3.10: *Phytophthora agathidicida* isolate NZFS 3770 sproduction inhibition curves using two fungicides
The error bars depict standard error based on three, biological replicates.

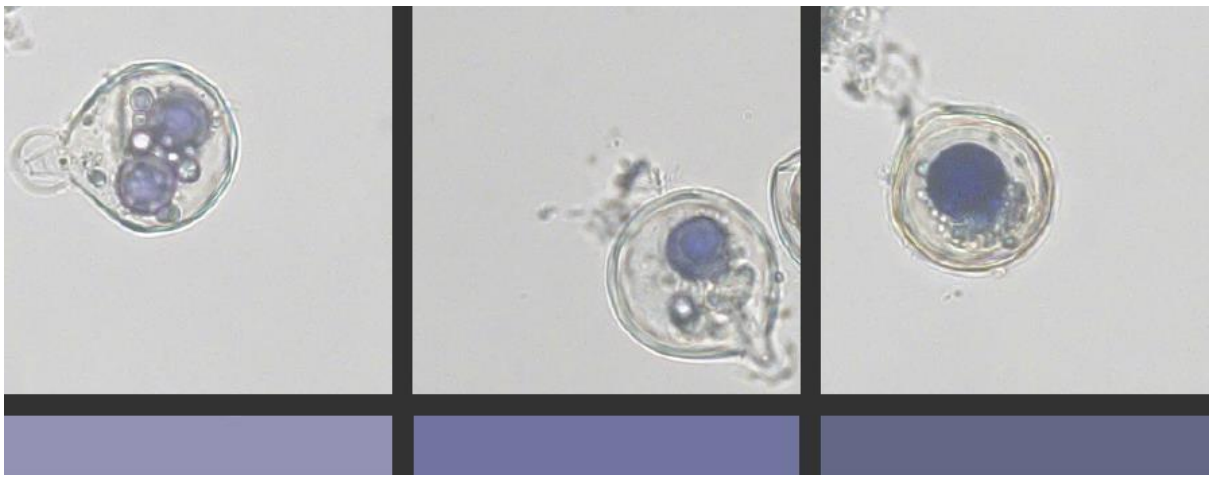

Figure 3.11: Variation in *Phytophthora agathidicida* oospores stained blue with the use of thiazolyl tetrazolium bromide (MTT)
Beneath each photograph is a representative colour swatch taken directly from the ooplast.

The difference in ooplast hue between the microscope eyepiece and camera was also documented during this assay (Figure 3.12). Specifically, oospores appeared to be more lightly stained and rose-hued when viewed using the microscope, whereas photographs using the Olympus DP74 microscope digital camera showed oospores with a darker, purple-hued ooplast.

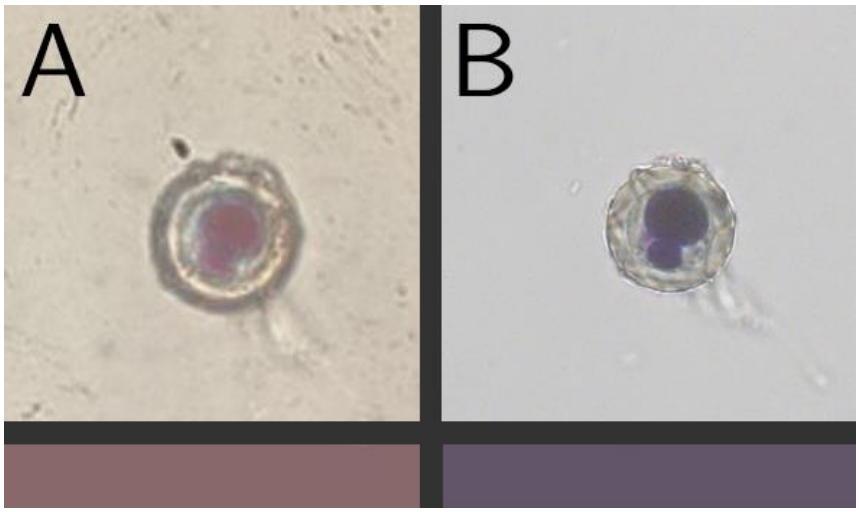

Figure 3.12: Difference in *Phytophthora agathidicida* oospore colouration using thiazolyl tetrazolium bromide (MTT) staining across different observational platforms.
a| microscope eyepiece and b| Olympus DP74 microscope digital camera.

3.4 Discussion

3.4.1 Overestimation of pathogen resistance using the disc diffusion assay

Although the disc diffusion assay has been successfully used to screen antimicrobial compounds against *P. agathidicida* mycelial growth (Lawrence et al., 2017, 2021), it did not accurately predict the inhibition potential of the fungicides and essential oils tested in this study. Specifically, the results from the disc diffusion assay significantly overestimated pathogen resistance to all anti-*Phytophthora*

candidates. This may have been due to unsuitable media used in the assay, as the original protocol calls for Mueller-Hilton agar (Bauer et al., 1966), which is a less viscous agar that allows for compounds to more easily diffuse (Nassar et al., 2019). However, it is more likely that this assay failed to accurately predict inhibition ranges due to a lack of compatibility of the test compounds, specifically with regards to their solubility. Disc diffusion assays are most suitable to hydrophilic compounds, whereas hydrophobic or amphipathic compounds may produce irregular or erroneous inhibition zones (Bonev et al., 2008). This has been previously reported for a range of antimicrobial compounds (Edelmann et al., 2007; Fehlberg et al., 2016; Maalej et al., 2011). The solubility of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin in water are 12.4, 2.8, 4.2, and 0.1749 mg/L, respectively, all of which are categorised as having low solubility (International Union of Pure and Applied Chemistry, 2020). Thus, the low solubility of these compounds likely interfered with their capacity to diffuse across the media, which affected the presence and size of inhibition zones.

3.4.2 Effect of land-use on *P. agathidicida* development

This study found that land-use and dominant vegetation type may influence *P. agathidicida* spore development, namely sporangia production. Specifically, soils extracts taken from two sites featuring native, old growth forest yielded a higher number of mature sporangia in comparison to pasture and exotic pine forests (Figure 3.8).

This potentially contrasts with a previous publication, which found that pasture and pine soils favoured initial *P. agathidicida* sporangia production (Lewis et al., 2019). That being said, the assay parameters between the two studies were quite different, both regarding the length of *P. agathidicida* exposure to soils and soil extracts (36 hours compared to a time series spanning eight days) as well as the media type (soils compared to dilute soil extracts). Also, both studies used a single landscape replicate, although Lewis et al. (2019) did use pseudoreplicates. Thus, additional land-use site replicates could be established to further elucidate the effect of land use on sporangia development.

3.4.3 Inhibition of *P. agathidicida* mycelial growth using essential oils

Thymus vulgaris essential oil was found to most effectively reduce *P. agathidicida* mycelial growth. This mirrors other studies that have found this essential oil to be a highly effective inhibitor across a wide variety of plant pathogens (Dewitte et al., 2019; Diánez et al., 2018; Sarkhosh et al., 2018). Comparing the EC_{50} value estimated in this study to those previously published using other *Phytophthora* spp. proved to be difficult, owing to the range of units reported (µg/mL, µL/mL, %, ppm), differences in thyme species extracted, and method variation (e.g., disc diffusion assay and agar dilution). That being said, the mycelial inhibition of *P. agathidicida* by *T. vulgaris* essential oil was similar to that of *P. infestans* (Najdabbasi et al., 2020), *P. palmivora* (Sarkhosh et al., 2018), *P. parasitica*

(Lu et al., 2013), and possibility that of *P. capsici* (Bi et al., 2012), which may have been reported with the incorrect units (Table 3.7).

Table 3.7. Inhibition of *Phytophthora* spp. mycelia with thyme (*Thymus vulgaris*) essential oil using the agar dilution method across multiple studies.

		EC_{50} value		
Phytophthora Species	Thyme species	µg/mL	µL/mL	Reference
P. capsici	*T. vulgaris*	≈7.17×10^{-2}		Bi et al., 2012
P. parasitica	*T. vulgaris*	≈3.6×10^{1}		Lu et al., 2013
P. infestans	*T. vulgaris*		≈5×10^{-1}	Najdabbasi et al., 2020
P. palmivora	*T. daenensis*		<1×10^{-1}	Sarkhosh et al., 2018
P. agathidicida	*T. vulgaris*	6.93×10^{1}	7.88×10^{-2}	Thurston, 2021 (Present study)

Perhaps unexpectedly, both indigenous plant essential oils, mānuka and kānuka, produced the lowest inhibition rates. This does not inherently conflict with the findings from Lawrence et al. (2019), which reported inhibition of *P. agathidicida* mycelia using kānuka crude extracts with an approximate EC_{50} value of 200 µg/mL, compared to 2,886.4 µg/mL reported here. The difference in EC_{50} values could be attributed to the presence, or lack thereof, of flavanone and β-triketone compounds found in mānuka and kānuka crude extracts compared to their corresponding essential oils. These specific classes of compounds have been reported to contribute significantly to antimicrobial activity (Lawrence et al., 2019; van Klink et al., 2005). One reason for the absence of these types of compounds could be due to the type of plant material extracted, as it has been found that inhibitory flavanones produced by kānuka are more concentrated in leaf tissue rather than roots (Lawrence et al., 2019), and it is not known what type of plant biomass was used to produce the essential oil. Alternatively, the essential oils used in this study may have been of poor quality in comparison to fresh extracts.

One caveat to using essential oils to manage environmental pathogens is that essential oils are subject to degradation when exposed to oxygen, light, and heat (Sherry et al., 2013). Liposome delivery mechanisms have been suggested as one possible application method for essential oils in the environment (Sherry et al., 2013). Liposome technology has already been shown to penetrate plant tissue to deliver micronutrients (Karny et al., 2018), increase the antimicrobial properties of some essential oils (Liolios et al., 2009), and treat disease in mammals (Shariare et al., 2020). The majority of liposomal delivery applications appear to be in the pharmaceutical and food science fields (Pedro et al., 2013), but it does not seem like they have been applied to plant pathogen models.

Alternatively, individual compounds identified by GC-MS could be screened against *P. agathidicida*. Compounds present either exclusively or in high concentrations in *T. vulgaris*, *M. alternifolia*, and *P. graveolens* essential oils were the subject of a brief literature review to assess whether they would be suitable candidates for further kauri dieback research. Eucalyptol (Sato et al., 2007), *p*-Cymene (Marchese et al., 2017), γ-Terpenine (Sato et al., 2007), borneol (Al-Farhan et al., 2010), thymol

(Falcone et al., 2005), terpenin-4-ol (Cordeiro et al., 2020), menthone (Kumar et al., 2011; Marei & Abdelgaleil, 2018), isomenthone (Kumar et al., 2011), citronellol (de Oliveira Pereira et al., 2015), and geraniol (de Oliveira Pereira et al., 2015) all had documented antimicrobial activity. Some of these may prove to be poor candidates for *P. agathidicida* inhibition, due to likely modes of inhibition involving mechanisms specific to true fungi. For example, both citronellol and geraniol inhibit ergosterol biosynthesis (de Oliveira Pereira et al., 2015), and thus could be ruled out for further *P. agathidicida* trials. Of these antimicrobial compounds, only thymol and menthone have been tested for anti-*Phytophthora* properties (Camele et al., 2012; Marei & Abdelgaleil, 2018), with both successfully inhibiting growth. Interestingly, multiple derivatives of thymol have also demonstrated anti-oomycete activity (Chen et al., 2020), which potentially highlights the potency of this chemical structure.

While mānuka and kānuka essential oils were least effective in inhibiting *P. agathidicida* mycelial growth, the bioactive potential of some of their constituents may warrant further investigation. Specifically, β-triketones, such as leptospermone and isoleptospermone, have antimicrobial activity (van Klink et al., 2005). In other studies, β-triketones accounted for approximately 31 ± 3.4% STE of the compounds in mānuka and kānuka essential oil (Park et al., 2017). In this study, however, their abundance was significantly lower, only approximately 2.7 ± 1.5% STE. This difference may in part have accounted for the low inhibition potential observed by mānuka and kānuka essential oils in this study.

3.4.4 Inhibition of *P. agathidicida* using fungicides

The EC_{50} values for mycelial inhibition of *P. agathidicida* using ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin all fell within the range of values that has been previously reported for other *Phytophthora* pathogens (Table 3.1). All four fungicides tested were found to inhibit *P. agathidicida* mycelial growth more effectively than those previously screened by Lawrence et al. (2017), although chlortetracycline hydrochloride had an EC_{50} value that was in the same order of magnitude as ethaboxam and fluopicolide. In addition to inhibiting mycelial growth, some fungicides also appeared to produce morphological changes at high concentrations. While this was outside the scope of this study, it would be beneficial to understand the changes in gene expression that are occurring. This has been done for other *Phytophthora* spp. through the use of RNA sequencing and transcriptome analysis (Hao et al., 2019; Zhang et al., 2017).

As has been previously reported, oxathiapiprolin most effectively inhibited *P. agathidicida* mycelial growth in comparison to the other fungicidal candidates (Table 3.1**,** Table 3.5**,** and Table 3.8). Zorvec® Enicade® was approximately two times less effective than the oxathiapiprolin analytical standard, which highlights the need to screen the other fungicides as commercial formulas, which would most likely be the form applied in an environmental setting. Ethaboxam (INTEGO™), fluopicolide (Presidio®,

Adorn®, and Infinito®), and mandipropamid (Revus®) have all been developed into commercial formulations, however, there is limited availability of these products in Aotearoa.

All four fungicides have been used successfully in field trials globally (Cerkauskas et al., 2015; Foster & Hausbeck, 2010; Ji & Csinos, 2015; Meyer & Hausbeck, 2013; Ren et al., 2018; Zhang et al., 2005), although it is not presently clear how the formulations would be applied to threatened or infected kauri. One benefit of phosphite is that it is translocated in both the xylem and phloem (Guest & Grant, 1991) and can be applied in a variety of ways (e.g. foliage or trunk sprays, soil dredges, injections) (Horner & Hough, 2014b). The manufacturer instructions for Zorvec® Enicade®, Presidio®, Infinito®, and Revus® all list foliar sprays as the application method, which would be impractical for kauri due to their size and may not be effective if applied to stem tissue or active lesions. That being said, experimentally, Zorvec™ (oxathiapiprolin) has been shown to effectively protect plants against *Phytophthora* pathogens when applied as a soil drench (Qu et al., 2016a). Presently, the only commercial fungicide whose manufacturer instructions recommend soil drenching is Adorn®.

Resistance management strategies would also need to be considered with the use of these fungicides, as all four are single-site inhibitors (Belisle et al., 2019; Hao et al., 2019). This could involve developing a fungicide rotation scheme, particularly with those belonging to different fungicide groups (Fungicide Resistance Action Committee, 2019; Gisi & Sierotzki, 2015), monitoring *P. agathidicida* regularly for signs of resistance development, or applying multiple fungicides with different modes of action at the same time. The latter option also offers the potential for synergism between fungicides, wherein a mixture of fungicides produces a greater inhibition effect than would be expected based on the inhibition effect of the fungicides individually. While the synergistic potential of *P. agathidicida* inhibitors has yet to be explored, previous studies of *Phytophthora* pathogens found increased disease inhibition when fungicides with different modes of action were applied as a mixture (Gisi et al., 1985; Grabski & Gisi, 1987; Samoucha & Cohen, 1986; Wang et al., 2014). It is worth noting that phosphite has shown synergistic potential with other fungicides (Gisi et al., 1985; Samoucha & Cohen, 1986), and thus the fungicides identified both by this study as well as the antimicrobial compounds presented by Lawrence et al. (2017) and (2021) should be considered as candidates for synergistic treatment with phosphite.

In additional to logistical considerations, suitability of these fungicides would also need to be vetted by tangata whenua|local Māori in accordance with their beliefs and practices. Presently, there is concern over the effect of run-off phosphite in native forests (Black & Dickie, 2016; Nuttall et al., 2010), and it is likely than any of these four fungicides would present similar concerns. In particular, aquatic toxicity as well as non-specific targeting in soil microbial communities are two considerations that would likely need to be investigated prior to field applications. With regards to the latter, previous

work has been conducted to characterise soil microbial community structures and function in kauri forests (Byers et al., 2020), the methods for which could be applied to soil fungicide trials as well.

3.4.5 Comparison of *P. agathidicida* mycelial inhibition across studies

Oxathiapiprolin is the most effective inhibitor of *P. agathidicida* mycelia to date, followed by the other fungicides trialled by this study (Table 3.8). Interestingly, a number of plant-derived natural products are more effective at inhibiting mycelial growth than some antimicrobial candidates tested by Lawrence et al. (2017). Essential oils, on the other hand, were generally the least effective at reducing mycelial growth.

3.4.6 Other fungicidal candidates

In the wake of the development and success of ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin, several of their derivatives have been designed and screened for anti-oomycete activity. Notably, derivatives of both fluopicolide and oxathiapiprolin have high anti-*Phytophthora* activity (Li et al., 2020; Wu et al., 2018; Zhang et al., 2014). Due to the success of fluopicolide and oxathiapiprolin on *P. agathidicida* mycelial growth inhibition, it would be worth screening these compounds as well once they become commercially available.

3.4.7 Alternate methods for assessing *P. agathidicida* sporangia and oospores

Determining the effect of fungicides on *P. agathidicida* sporangia formation, zoospore motility, and oospore viability would improve kauri dieback management options. For example, in this study, ethaboxam was found to inhibit sporangia production more effectively than mycelial growth, which could inform potential fungicide application methods. Unfortunately, the methods used in this study to assess *P. agathidicida* life cycle stages other than mycelial growth were highly variable or did not detect a treatment effect (e.g., no effect on viability with increasing fungicide concentration). Thus, alternate methods for producing and assessing these life cycle stages should be considered.

Table 3.8: Compilation of EC_{50} values for *P. agathidicida* mycelial inhibition using fungicides and plant-based compounds or extracts.

The EC_{50} values are sorted from lowest to highest concentration required for 50% inhibition. Inhibitors originating from the Lawrence et al., (2021) study are natural products for which neither the CAS number nor given name were provided, thus, they are described by the number assigned to them in that publication.

Phytophthora agathidicida inhibitor	EC_{50} (µg/mL)
Oxathiapiprolin[1,2]	$1.0\text{-}1.7 \times 10^{-4}$
Mandipropamid[1]	2.22×10^{-2}
Ethaboxam[1]	1.04×10^{-1}
Fluopicolide[1]	$3.89 \times 10^{--1}$
Chlortetracycline hydrochloride[4]	7.90×10^{-1}
Compound 20[3]	8.92×10^{-1}
Compound 15[3]	2.35
Compound 2[3]	3.02
Benzathonium chloride[4]	3.20
Compound 21[3]	3.65
Phosphorus acid[6]	4
Compound 3[3]	4.28
Paromomycin sulphate[4]	4.30
Compound 19[3]	5.73
Copper (II) chloride[4]	6.80
Compound 13[3]	7.24
Compound 8[3]	8.03
Compound 14[3]	8.20
Cooper (II) sulphate[4]	9.00
Compound 4[3]	9.53
Compound 12[3]	1.02×10^{1}
Compound 17[3]	1.05×10^{1}
Compound 9[3]	1.15×10^{1}
Compound 5[3]	1.51×10^{1}
Compound 1[3]	2.37×10^{1}
Compound 11[3]	2.43×10^{1}
Compound 10[3]	2.65×10^{1}
Kanamycin sulphate[4]	2.80×10^{1}
Thyme (*Thymus vulgaris*) essential oil[1]	6.93×10^{1}
Compound 16[2]	7.40×10^{1}
5,7-Dihydroxy-6-methylflavanone 1[4]	1.00×10^{2}
Neomycin trisulfate[4]	1.80×10^{2}
Rose geranium (*Pelargonium graveolens*) essential oil[1]	1.86×10^{2}
D-cycloserine[4]	1.90×10^{2}
Tea tree (*Melaleuca alternifolial)* essential oil[1]	1.09×10^{3}
Kānuka (*Kunzea ericoides*) essential oil[1]	2.89×10^{3}

[1]**Present study**

[2]Lacey et al., 2021a

[3]Lawrence et al., 2021

[4]Lawrence et al., 2019

[5]Lawrence et al., 2017

[6]Horner & Hough, 2013

3.4.7.1 Sporangia production

Phytophthora agathidicida sporangia production was inconsistent both within and across assays. While the pathogen was passaged through pear every eight growth cycles, as per previous finding regarding pathogenicity (Armstrong, 2018), it is possible that the isolates used in this study lost fitness over a shorter time frame and thus needed to be passaged more frequently. Alternatively, repeated mycelium inoculation may have selected for reduced sporangia production capacity over time, which would not necessarily be re-stimulated with pear passaging, as *P. agathidicida* mycelium is capable of causing disease in pear tissue on its own. Another contributing factor to the inconsistent sporangia production may have stemmed from the incubation media used. The concentration of soil extracts or pond water needed to induce sporangia production varies seasonally (Armstrong, 2018). Thus, while filtered, non-sterile pond water was shown to enhance sporangia production over other incubation media in an initial assay, it is possible that it was less potent when used in the fungicide trials a month later.

It is also possible that a different sporangia production method would have led to more consistent and replicable results. The sporangia production method used in this experiment was based off of previous *P. agathidicida* research (Lawrence et al., 2017, 2019), in which sporangia formation was induced as a means of producing zoospores. Hence, this method optimises zoospore counts through the stimulation of fresh mycelial production using carrot broth. This differs to studies that have focused on the effect of fungicides on sporangia production in other *Phytophthora* spp., which do not stimulate mycelial production and instead incubate agar plugs containing active mycelia directly with sterile soil extracts (Bittner & Mila, 2016; Ji & Csinos, 2015; Miao et al., 2016b; Qu et al., 2016b). These methods also differed in that they used between three and ten pseudoreplicate agar plugs in each of the three biological treatments, which would have acted to lower variability.

3.4.7.2 Oospore viability

Isolated oospores suspended in increasing concentrations of oxathiapiprolin over a 48-hour period maintained their viability. It is possible that the concentration range used, which was informed by the mycelial inhibition assay, was not appropriate and that *P. agathidicida* oospores require much higher concentrations to lose viability. Alternatively, the method used, which was based on previous *P. agathidicida* research (Dick & Kimberley, 2013), may not be the most suitable for determining oospore viability. Instead, germination rates could be determined for isolated oospores exposed to either kauri root extracts or agar media amended with fungicides (Beever et al., 2010; Duncan, 1985; Lacey et al., 2021a), which are methods that have both been validated with *Phytophthora* oospores.

The presence of blue stained oospores with MTT was unexpected based on the results observed in Section 2.3.3. Following the *P. agathidicida* classifications outlined by Dick & Kimberly (2013) these

oospores would be considered non-viable. However, many other papers classify blue oospores as active and rose-hued or purple oospores as dormant (Beakes et al., 1986; Jiang & Erwin, 1990; Medina & Platt, 1999; Sutherland & Cohen, 1983). If these guidelines were instead followed, it would change the results previously listed in in Section 2.3.3 in that all purple stained spores, regardless of stain intensity, would be classified as dormant. Unfortunately, this issue would likely only be elucidated by a more comprehensive germination study to properly correlate MTT vital staining with spore germinability or by developing alternative staining protocols that more clearly distinguish viable and non-viable spores. It appears that a fluorescence-based oospore viability study has been recently developed for *P. agathidicida* (Fairhurst et al., 2021), however this method has not yet been peer reviewed.

3.5 Conclusion

This study identified fungicides and essential oils that inhibited *P. agathidicida* mycelial growth. Oxathiapiprolin inhibited *P. agathidicida* mycelial growth more than any previously tested fungicide or antimicrobial compound (Table 3.8). While the essential oils possessed some of the lowest inhibition rates (Table 3.8), a number of potential *P. agathidicida* inhibitory compounds were identified by GC-MS (Table 3.6), which warrant screening. Based on previously published studies, thymol and its derivatives, menthone, leptospermone, and isoleptospermone are the most promising candidates found by this study.

Although EC_{50} values for ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin were successful estimated for *P. agathidicida* mycelial inhibition, further work is needed to determine their effect on other life cycle stages of the pathogen. This may be accomplished by implementing the suggestions listed in Section 3.4.7. Additionally, a few recent pre-prints contain relevant *P. agathidicida* screening and viability protocols (Fairhurst et al., 2021; Lacey et al., 2021a), which may inform future studies.

Despite the promise these fungicides hold for kauri dieback management, this study represents the first of many that would need to be conducted for them to be applied as a treatment. This would likely include greenhouse studies, such as those conducted with phosphite (Horner & Hough, 2013), and run-off environmental effects, as well as issues brought up through mana whenua|local Māori consultations to identify persistent cultural concerns over applications.

Chapter 4:

Detection of *Phytophthora agathidicida* from environmental samples

4.1 Introduction

4.1.1 Difficulties applying molecular detection methods to soils

Only ≈1% of soil microbes can be cultured, therefore, molecular methods are most commonly used to conduct community analysis or detect specific microbes (Vartoukian et al., 2010). However, molecular methods are hampered by the presence of various soil components that inhibit cell lysis, DNA polymerase activity, and PCR amplification (Wilson, 1997). These include, but are not limited to, bile salts, bilirubin, clay particles, fulvic acids, humic acids, metal ions, phenolic compounds, polysaccharides, and urobilinogens (Crecchio & Stotzky, 1998; Matheson et al., 2010; Watson & Blackwell, 2000; Wilson, 1997; Yeates et al., 1998). Of these, humic acids have been noted as being the most problematic inhibitor (Watson & Blackwell, 2000), which is in part due to the fact that they inhibits both DNA polymerase and PCR amplification (Matheson et al., 2010).

Kauri grow on clay soils (Jongkind & Buurman, 2006; Molloy, 1998) and input humic acids into the soil via kauri leaf decomposition (Molloy, 1998), both of which have likely contributed to reduced pathogen detection using molecular methods with kauri forest soils (Than et al., 2013).

4.1.2 Challenges with detecting *Phytophthora* pathogens from soil

Phytophthora pathogens belong to the 99% of microbes that are generally considered to be 'unculturable', as the background soil microbial community often antagonises or outgrows *Phytophthora* spp. (Tsao, 1983). While molecular methods have been developed for detecting *Phytophthora* pathogens (Bienapfl et al., 2011; Bilodeau et al., 2007; Zhang et al., 2006), there are persistent issues with their efficacy.

Certain *Phytophthora* life stages are difficult to detect. For example, thick-walled oospores are more resistant to cell lysis (Lees et al., 2012), which can lead to lower recovery rates using molecular methods. Oospores also germinate more slowly than sporangia, and thus require a longer incubation period if using soil baiting or hybrid detection methods (Dick, 1966).

Another general issue with pathogen detection is that their presence in environmental samples is typically low. This may be remediated partially through processing multiple sub-samples (Brierley et al., 2009) or analysing larger samples (Beever et al., 2010).

4.1.3 Importance of accurate and efficient detection methods

Accurate and timely detection of *P. agathidicida* is critical as it allows for well-informed treatment options, including both targeted applications of fungicides or rongoā as well as identifying and prioritizing kauri in need of boardwalk upgrades or rāhui. What follows is an overview of the methods that have been developed for *P. agathidicida* detection and a critical analysis of the efficacy of these methods.

4.1.3.1 Visual surveillance

Visual surveillance, or ground truthing studies, for *P. agathidicida* presence have typically assessed both kauri canopy health and the severity of kauri stem lesions. However, the correlation between canopy health and lesion presence is variable. For example, in one study, approximately 15% of kauri with healthy canopies had basal lesions present whereas 57% of kauri with dead canopies had no lesions documented (Waipara et al., 2013). Previous field assessments have found that ground trothing surveys were only able to accurately diagnose *P. agathidicida* presence approximately 33% of the time (Waipara et al., 2013). This is in part attributed to misdiagnosis of resinosis (Beever et al., 2010), false negatives due to delays between root infection and above ground symptoms (Beever et al., 2010), and differences between symptoms across sites (Beauchamp, 2013). Unfortunately, these surveys risk further spreading *P. agathidicida* through the movement of soils associated with shoes, tyre treads, and even sampling equipment (Hill et al., 2017; Pau'uvale et al., 2011). For this reason, ground truthing surveys are generally limited in scale and conducted in sites immediately adjacent to tracks, which subsequently restricts the areas that they can be conducted (Jamieson et al., 2014).

4.1.3.2 Remote sensing

Aerial photography and remote sensing have recently immerged as alternative methods for visual assessments. Detection using aerial photographic detection is both cost and time effective but can only be used to detect advanced dieback symptoms (Jamieson et al., 2014). Remote sensing allows aerial photography data to be combined with LiDAR data, satellite imagery, and hyperspectral images. This method has been shown to be able to distinguish kauri from other canopy species with high accuracy and has the potential to detect early dieback symptoms (Meiforth et al., 2019, 2020). However, using hyperspectral images is currently cost prohibitive for regular use (Meiforth, 2019) and neither method can distinguish between *P. agathidicida* infection and the presence of other plant diseases or environmental stressors (Jamieson et al., 2014; Meiforth et al., 2020).

4.1.3.3 Tissue sampling

Phytophthora agathidicida has been successfully isolated from diseased kauri cork cambium, sapwood, and root tissue (Beever et al., 2010; Gadgil, 1974; Waipara et al., 2013). In one study, detection of *P. agathidicida* was higher in diseased plant tissue compared to soil samples (Gadgil, 1974). That being said, lesions in kauri may also present without recoverable *P. agathidicida*. This could be due to alternative mechanisms of lesion occurrence and resinosis production (see Section 1.3.1) or instances in which *P. agathidicida* caused a primary infection that gave way for a secondary pathogen to take hold and cause more significant damage (Beever et al., 2010).

The potential benefits of sampling lesions include rapid field testing and the possibility for reduced assay costs (Beever et al., 2010). While sampling cambium and sapwood tissue poses little chance for long-term damage to the tree (Beever et al., 2010), it is not a popular sampling method as it is viewed by the public as invasive and likely to cause further infection (Waipara et al., 2013).

4.1.3.4 Lateral flow devices

Lateral flow devices (LFD) are portable, serological kits that detects specific antigens from a sample in a matter of minutes (Martin et al., 2012). To date, there has been one LFD developed for the *Phytophthora* genus — the Pocket Diagnostic Test Kit™. This LFD is suitable for testing diseased plant material, but not soil, and has been shown to be effective in detecting *Phytophthora* from kauri lesions (Beever et al., 2010; Waipara et al., 2013). While the current LFD used in kauri dieback research is not specific to *P. agathidicida*, there is precedent for the development of species-specific LFD for *Phytophthora* pathogens (Dai et al., 2019). Furthermore, LFD membranes that test positive for pathogen presence have been used in TaqMan real-time PCR and LAMP assays, which could bypass the need to extract DNA entirely and consequently allow for more rapid *P. agathidicida* detection (Tomlinson et al., 2010a; Tomlinson et al., 2010b).

4.1.3.5 Scent detection canines

Scent detection canines have been trained to detect various pathogens and diseases in both plants and mammals, including lung cancer in humans (McCulloch et al., 2012), methicillin-resistant *Staphylococcus aureus* (MRSA) (Koivusalo et al., 2017), laurel wilt disease in avocado trees (Mendel et al., 2018), and *Phytophthora* pathogens from soil, water, and plant material (Swiecki et al., 2018).

Recently, scent detection canines have been applied to kauri dieback. In 2015, one scent detection canine was able to positively detect *P. agathidicida* with 93.5% accuracy and discern it from negative samples, either non-inoculated or inoculated with *P. cinnamomi* or *P. multivora*, with 96% specificity (Bassett, 2016). While this initial pilot was promising, this particular canine did not express interest in more complex samples (e.g., root and soil samples) (Bassett, 2016). As two other canines have now been trained and shown to detect *P. agathidicida* from kauri bark and soil samples (Bell, 2021), it

appears that the initial issues with the pilot study reflect the dog's temperament and training rather than an inability of canines to detect kauri dieback in the environment.

Despite the most recent success with using scent detection canines, it is worth pointing out that there is no available data as to their accuracy or specificity and that these dogs require further training before they are able to be deployed with handlers in the environment (Kerr-Lazenby, 2021).

4.1.3.6 Soil Baiting

Phytophthora species are notoriously difficult to isolate from environmental samples, owing to their slow growth rate in comparison to the background microbial community (Beever et al., 2010; Erwin & Ribeiro, 1996). To address this issue, baiting methods have been used for over half a century to isolate *Phytophthora* pathogens. These methods utilise a highly susceptible host, referred to as a "bait", that is readily infected by the pathogen and can be subsequently cultured on selective media (Erwin & Ribeiro, 1996).

The efficacy of baiting assays relies on the temperature, light level, water quality, spores present in the sample, and the plant species and part (e.g., leaves, roots, whole specimen, etc.) used (Beever et al., 2010; Dick, 1966; Singh et al., 2017; Tsao, 1983). Baiting assays typically use large quantities of soil, which can act to counter detection issues associated with soil heterogeneity and uneven inoculum distribution (Beever et al., 2010; Bellgard et al., 2013). As soil baiting requires the presence of viable, infectious spores, it only detects living *P. agathidicida*, as opposed to relic DNA (Bradshaw et al., 2019). It has been shown that *P. agathidicida* can be baited from soil samples as small as 0.5 g, although larger samples have greater recovery rates (Beever et al., 2010). Thus, a 100 g sample size is recommended by the standard operating procedure (SOP) (Waipara et al., 2013), although the use of soil quantities this large can act to reduce the number of samples that can be processed at any given time. A variety of plant baits for *P. agathidicida* were assessed at Scion, Plant & Food, and Landcare, of which lupin radicles were found to be the most susceptible (Beever et al., 2010). That being said, these baits only had a successful infection rate of 78 ± 16% with zoospores and 41 ± 8.4% with naturally infested soils (Beever et al., 2010).

Despite the widespread use of soil baiting to detect *P. agathidicida*, this method has had issues with efficacy and reproducibility. The rate of successful *P. agathidicida* recovery using optimised soil baiting methods from environmental samples taken from kauri dieback areas ranges from 10 to 62% (Bellgard et al., 2013; McDougal et al., 2014; Singh et al., 2017). The detection rate is slightly higher for lab inoculated soils, ranging from 20 to 80% (Beever et al., 2010; McDougal et al., 2014). There is also considerable variation in positive detection rates between different testing organisations, ranging from 25 to 71% agreement (Beauchamp, 2013; Beever et al., 2010; Bellgard et al., 2011; McDougal et al., 2014). A recent study suggested that low recovery rates may be due to inadequate assay

optimisation, which allows for other, faster-growing *Phytophthora* and *Pythium* species to be cultured instead of *P. agathidicida* (Winkworth et al., 2020). Furthermore, this process is intensive and time-consuming — taking between 15 and 25 days to isolate pathogens (Beever et al., 2010; McDougal et al., 2014; Winkworth et al., 2020) and subsequent morphological studies require the use of extensive taxonomic keys, which can still lead to misidentification (Than et al., 2013).

Due to the low throughput capacity, time intensiveness, and cost, soil baiting for *P. agathidicida* is typically only conducted to confirm the disease when physical symptoms have presented (Winkworth et al., 2020). This restricts the ability of mana whenua kaitiaki|environmental technicians to manage kauri dieback spread on a local and regional level and limits our understanding of the current distribution of the pathogen.

4.1.3.7 Molecular sequencing using polymerase chain reaction (PCR)

The internal transcribed spacer (ITS) region has historically been used for sequence-based species identification of fungi (Martin et al., 2012). While *Phytophthora* are not true fungi, the ITS region has been similarly used for species identification and the construction of phylogenetic trees within this genus (Cooke et al., 2000). *Phytophthora agathidicida* has an identical ITS sequence to *P. castaneae* (≡ P. katsurae, nom. Illegit (Pennycook, 2012)), but can be distinguished by its cytochrome oxidase 1 (COX1), enolase (ENL), and NADH dehydrogenase subunit 1 (ND1) sequences (Weir et al., 2015). As *P. castaneae* is not present in Aotearoa (Scott & Williams, 2014; Weir et al., 2015), ITS sequencing can be used to identify *P. agathidicida* reliably.

Polymerase chain reaction (PCR) is a technique used to amplify targeted regions of DNA. This method detects DNA from *Phytophthora* spores of different life cycle stages and from species with low abundance in complex environmental samples (Scibetta et al., 2012). The real-time PCR (qPCR) assay developed for *P. agathidicida* shows cross reactivity with *P. castaneae* and *P. novaeguineae*, however, as it does not amplify DNA from other *Phytophthora* and *Pythium* species present in Aotearoa (Bellgard et al., 2013; Scott & Williams, 2014) it is considered to be highly specific. The detection threshold using this assay is 2 fg DNA from pure cultures and 20 fg DNA from soil samples (Than et al., 2013) The reverse PCR primer was originally incorrectly published (Than et al., 2013) and the correct sequence can be found in a later report (McDougal et al., 2014).

There are a few notable issues with the qPCR assay developed for *P. agathidicida*. The published method calls for handshaking samples with one-inch steel ball bearings to lyse cells (Than et al., 2013), which has been reported to cause catastrophic sample loss and likely leads to inconsistencies in extraction efficacy (McDougal et al., 2014). Furthermore, it appears that the current qPCR assay is not optimised for detecting the pathogen in soil samples (Bellgard et al., 2013; McDougal et al., 2014; Singh et al., 2017). This may be attributed to the fact that extracted soils contain PCR inhibitors, which

impede amplification (Singh et al., 2017). In particular, kauri forest soils contain high levels of humic acids, which can cause template inhibition, prevent DNA from being amplified, or directly inhibit the activity of DNA polymerase (Matheson et al., 2010; Picard et al., 1992). Finally, PCR assays generally do not distinguish between infectious inoculum and relic DNA, causing detection rates to be overinflated when testing environmental samples (Carini et al., 2016; Scibetta et al., 2012; Singh et al., 2017).

4.1.3.8 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is another molecular technique that amplifies DNA, but differs from PCR in that it uses a greater number of primer sets and therefore targets more DNA regions, runs at a constant temperature rather than thermal cycling, utilises polymerases that are more resistant to inhibition, can be conducted and analysed with less complex instruments, and in some instances has been shown to be more sensitive than PCR (Li et al., 2017; Notomi et al., 2015).

A LAMP assay was recently developed for *P. agathidicida* that uses a nucleotide sequence in apocytochrome b (*cob*) as the target sequence for amplification (Winkworth et al., 2020). This assay is more specific than its PCR counterpart (i.e., does not cross-react with *P. castaneae*) and was also found to have a significantly lower detection limit (Winkworth et al., 2020). As LAMP products can be detected by turbidity and colourimetry assays, there is the possibility of developing a field assay for *P. agathidicida* detection (Winkworth et al., 2020). This LAMP assay has not yet been validated on environmental samples, and so it is not known whether it will encounter similar issues regarding amplification inhibition.

4.1.3.9 Hybrid methods

Hybrid methods utilise a susceptible host to bait out viable pathogen from environmental samples, after which the baits themselves or the pure cultures isolated from them are sequenced using molecular methods (Singh et al., 2017; Winkworth et al., 2020). This approach has been recommended for both PCR-based analyses (Khaliq et al., 2018; Singh et al., 2017) and LAMP assays (Winkworth et al., 2020).

This method, along with the extended soil baiting assay that performs morphological studies on the baited isolates, are currently the preferred method for *P. agathidicida* detection from soils (Winkworth et al., 2020). While hybrid methods reduce assay time and costs, the baiting process still takes between 8 and 14 days and may require the use of specialised equipment, consumables, and skilled staff.

4.1.3.10 Lipid profiling

Lipid profiling, either using fatty acid methyl ester (FAME) or phospholipid fatty acid (PLFA) analysis, uses extracted lipids as biomarkers to identify microbial communities or determine soil health

(Cavigelli et al., 1995; Li et al., 2020). This type of profiling has been used to identify *Phytophthora* pathogens from soils samples, even down to a species level (Duan et al., 2013; Larkin & Groves, 2003; Yousef et al., 2012).

Efforts to develop detection methods for kauri dieback using lipid profiling did not identify unique *P. agathidicida* biomarkers, however, the relative quantities of 20:4ω6 and 20:5ω3 may be used to detect the overall presence of *Phytophthora* pathogens from soil samples (Lacey et al., 2021b).

4.1.3.11 Commercial DNA extraction kits

Commercial DNA extraction kits refer to products that supply the consumables and reagents needed for rapid extraction and purification of DNA from different types of samples. The efficacy of DNA extraction kits varies between products, which can result from differences between kits materials (e.g., use of lysis agents or employment of agitation methods) or inherent characteristics of the samples themselves (e.g., soil pH or organic matter content) (Dineen et al., 2010; Fredricks et al., 2005). For soil samples, the most popular DNA extraction kits used are DNeasy® PowerSoil® (12888-100, Qiagen), which process up to 0.25 g sample, and DNeasy® PowerMax® (12988-10, Qiagen), which process up to 10 g sample (Lear et al., 2018).

To date, only the DNeasy® PowerSoil® extraction kit has been used on environmental samples to detect for *P. agathidicida* (Byers et al., 2020; Schwendenmann & Michalzik, 2019; Singh et al., 2017), which was found to have a detection threshold of 5.21 fg/µL (Singh et al., 2017).

4.1.4 Comparison of *P. agathidicida* detection methods

Numerous studies have now been conducted that utilise a combination of ground truthing, soil baiting, and molecular methods to analyse the same soil samples and sites for kauri dieback. From these reports, it is possible to determine how frequently the results of these techniques agree with one another, which is critically analysed below.

4.1.4.1 Soil baiting vs. ground truthing surveillance

Ground truthing surveillance for kauri dieback overestimates the co-occurrence of *P. agathidicida* with possible dieback symptoms, with a 40 to 51% false positive rate (Beauchamp, 2013; Bellgard et al., 2013; Waipara et al., 2013). This could be due to non-ubiquitous disease symptoms (Beauchamp, 2013) or instances of late-disease progression, in which the vast majority of the pathogen loading is in plant biomass, as opposed to the soil.

For trees in good condition (e.g., dense foliage with < 10% resin coverage on the trunk), ground truthing surveys fail to detect kauri dieback between 9 to 18% of the time (Beauchamp, 2013; Bellgard et al., 2013). This may be attributed to the undefined latency period of *P. agathidicida* (Bradshaw et al.,

2019), in which the pathogen may be present in both soils and kauri root structures with no visible aboveground biomass symptoms.

4.1.4.2 Soil baiting vs. molecular detection methods

Generally, direct comparisons of molecular methods against soil baiting show that molecular methods (PCR or LAMP) detect *P. agathidicida* in soils samples at higher rates when compared to soil baiting (Table 4.1). This could be due to the presence of either relic DNA from non-viable spores, *P. agathidicida* spores that are viable but not conducive to producing zoospores, or faster growing *Phytophthora* or *Pythium* spp. that outcompete *P. agathidicida* in the extended soil assay (Singh et al., 2017; Winkworth et al., 2020).

There is relatively low positive detection agreement between methods (37 ± 8.3% STE). This cannot be exclusively attributed to the higher number of positive detection rates using molecular methods, as *P. agathidicida* was detected only via soil baiting approximately 22% of the time.

Table 4.1: Comparison of positive *Phytophthora agathidicida* detection rates between soil baiting and quantitative PCR

	Number of positive *P. agathidicida* detections				
Samples tested	Soil baiting	qPCR	Total number of positive samples	Percent positive agreement[1]	Reference
115	29	20	29	69%	Bellgard et al., 2011
40	11	16	20	35%	Bellgard et al., 2013
26[2]	7	9	13	23%	McDougal et al., 2014
26[2]	3	9	9	33%	McDougal et al., 2014
44	27	41	N/A	N/A	Singh et al., 2017
14	2	8	8	25%	Winkworth et al., 2020

[1]Percent agreement was calculated by dividing the number of instances in which soil baiting and molecular methods both positively detected *P. agathidicida* by the total number of samples that tested positive by at least one method.

[2] This sample set was tested by two different research organisations, thus, it warranted two entries in this table.

4.1.4.3 LAMP vs. hybrid methods

To date, only one study has compared *P. agathidicida* detection rates between qPCR and LAMP hybrid methods. Both methods detected *P. agathidicida* from pure cultures without cross-reacting with other *Phytophthora* pathogens (Winkworth et al., 2020), and detection using LAMP had a similar detection threshold to what has previously been published for qPCR (Than et al., 2013).

4.1.5 Standard operating procedure for kauri dieback detection

Presently, *P. agathidicida* is most commonly detected from environmental samples through soil baiting followed by morphological assessment of cultures (extended soil bioassay) or molecular identification (hybrid methods) (Winkworth et al., 2020).

4.1.6 Proposed study

This study aims to improve molecular methods for *P. agathidicida* detection in environmental samples. First, the extended soil bioassay followed by molecular identification of select isolates was applied to ten soils samples collected near kauri to screen for *P. agathidicida* presence. Following this, the molecular methods previously described by Than et al. (2013) and McDougal et al. (2014) were altered to standardise the extraction process, increase DNA extraction by enhancing cell lysis, and improve DNA purity by reducing the co-precipitation of humic acids with DNA. This was done by utilising an automatic shaker in addition to handshaking, using a variety of ball bearing sizes and materials, and adding chemical flocculants to the extraction buffer. Inoculated soils were also extracted using DNeasy® PowerSoil® and PowerMax® kits, which enabled the comparison between manual and commercial extraction methods. All methods were compared based on the total quantity of DNA extracted (ng/µL), spectrophotometry absorbance ratios (nm/nm) of 260/280 and 260/230, and detection of *P. agathidicida* using PCR.

Originally, the detection threshold for both the optimised manual DNA extraction protocol and the commercial extraction kits were to be establish through a qPCR assay, which would have utilised three, ten-fold *P. agathidicida* inoculum loads with kauri soils with additional replication (5-10 per method). Due to a month-long delay in receiving TaqMan™ Environmental Master Mix, this component of the project could not be completed within the timeframe of this study.

4.2 Methods

4.2.1 Soil samples

The ten soil samples used for the extended soil bioassay and hybrid methods were collected by Adrian Peachy from Northland Regional Council following the MPI kauri dieback (KDB) standard operating procedure (SOP) guidelines. Each sample was collected in collaboration with either an intermediate or secondary school and was taken close to kauri that have not previously tested positive for *P. agathidicida* (Table 4.2). The soils samples were all smaller than what is typically baited according to published literature (Than et al., 2013; Winkworth et al., 2020), although the soil baiting SOP for *P. agathidicida* validates soil baiting using sample quantities as low as 20 g (Beever et al., 2010; McDougal et al., 2014).

The soil used for optimising manual DNA extraction methods and comparing its efficacy to that of commercial extraction kits was collected by Lee Hill from BioSense on 2020-05-25. The soil was collected from Titirangi, Tāmaki Makaurau within the rohe|area of Te Kawerau a Maki Iwi, and assumed to be free from *P. agathidicida* due to lack of symptomology in kauri. Soil from this area is characterised as brown granular clay soil (Martindale et al., 2018).

Upon receiving, all soils were stored in sealed, plastic bags at 4°C until they were further processed.

Table 4.2: List of soil samples and dry weight that were used in the extended soil bioassay and hybrid methods
Hatched cells denote samples for which the location was not given.

Sample ID	Location	Lab ID	Wet weight (g)
Kamo high school	Whangārei	1	89.107
Huanui college	Glenbervie	2	91.859
Frank H Sample 2		3	50.829
Frank H Sample 1		4	87.238
Tikipunga high school	Whangārei	5	84.570
Whareora Sample 2	Whareora	6	28.558
WBHS	Whangārei	7	82.355
WGHS	Whangārei	8	46.405
Frank H Sample 3		9	24.328
Whareora Sample 1	Whareora	10	50.078

4.2.2 Soil baiting

Soil samples (Table 4.2) were subjected to the soil baiting protocol described by Beever et al. (2010).

The entirety of each sample was manually processed by hand to break up large aggregates and remove plant debris and rocks. The samples were then weighed separately into appropriately sized bait containers and dried at room temperature for up to three days. On the fourth day, the samples were sprayed with RO water until the soil's surface had a visible sheen, but not so much that standing water was present. The containers were then loosely covered at room temperature for a four-day moist incubation.

During the moist incubation, lupin (*Lupinus angustifolius*) (F4260, King Seeds) baits were prepared. The assay requires five lupin seedlings for each sample, which were prepared in triplicate to ensure sufficient germination rates. Lupin seeds were mixed with wet, but not soaking, vermiculite and then covered loosely with aluminium foil at room temperature.

After the moist incubation, the samples were carefully flooded, minimising soil disturbance, with 500 mL RO water. Five germinated lupin seedlings were added to each container on an ethanol sterilised

polystyrene float, ensuring that the radicle was in contact with the water, but not the soil (Figure 4.1). Five Himalayan cedar (*Cedrus deodara*) needles were also floated directly on the surface of the water.

Figure 4.1: Soil baiting *Phytophthora agathidicida* from soils using lupin seedlings and cedar needles as baits

The baits were allowed to become infected over a two-day period, after which they were collected. The 1 cm tip of the lupin radicle as well as the whole cedar needle was harvested, surface sterilised with 70% ethanol for 30 seconds, and then rinsed twice with RO water. Baits were then dried, cut transversely, and placed on selective media for culturing.

All baits were assessed multiple times over a two to five day period for sub-culturing. If hyphal growth was observed emanating from a bait (Figure 4.2), a section of agar containing the mycelia was removed and transferred to selective media. If needed, this process was repeated until a pure culture was obtained.

Figure 4.2: Microbial growth from lupin and cedar baits used in a soil baiting assay
A| Likely bacterial growth from lupin radicle baits. B| Hyphal growth emanating from cedar baits.

Pure cultures were maintained on 20% cV8 or submerged in sterile 20% V8 broth (See Section 2.2.2 for media recipes) to produce a mycelial mat free from agar.

4.2.3 Microscopy study of baited isolates

Spore and hyphae morphology was documented with microscopy following the agar block smear preparations previously described in Section 2.2.8. Two agar plugs were taken from each pure isolate grown on 20% cV8, one from the leading edge of hyphal growth and another from the oldest growth. The purpose of this was to increase the likelihood of observing multiple lifecycle stages, and consequently spore types, for each isolate.

Each stained agar plug was observed at 40x magnification and photographed with an Olympus DP74 microscope digital camera. Spores and hyphae were evaluated using identification keys and criteria (Abad et al., 2019; Erwin & Ribeiro, 1996; Webster & Weber, 2007).

4.2.4 DNA extraction from pure isolates

For baited isolates that were either morphologically consistent with *Phytophthora* pathogens or for which there was not enough evidence to rule them out, genetic analysis was used to confirm species identity.

DNA was extracted from pure isolates grown in 20% V8 broth using DNeasy® UltraClean® Microbial kits. The only deviation from the manual instructions was that rather than collecting 1.8 mL microbial culture in liquid to extract, a flame sterilised metal loop was used collect hyphae and then transfer it

into the collection tube (Figure 4.3). After successful extraction and purification of DNA, all samples were stored at -20°C.

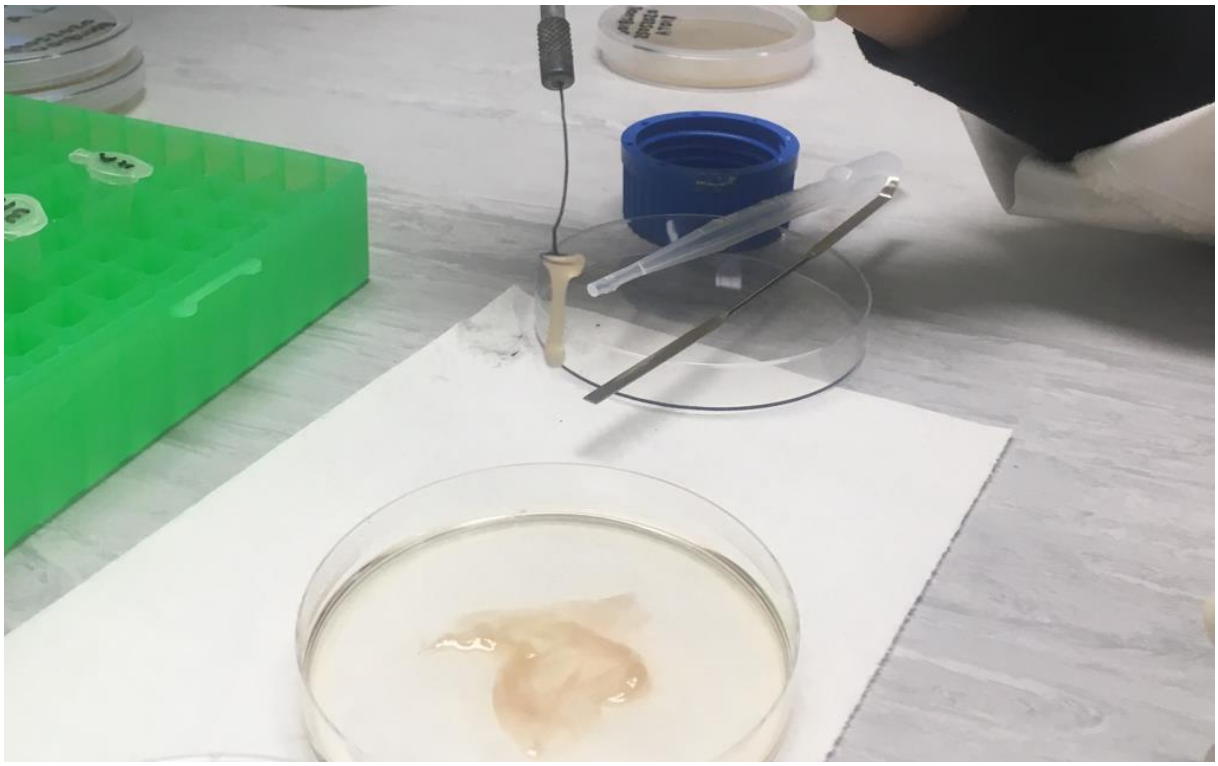

Figure 4.3: Collection of hyphae for DNA extraction using DNeasy® UltraClean® Microbial kits

4.2.5 Polymerase chain reaction (PCR) using general *Phytophthora* primers

DNA extracts from baited isolates were amplified using ITS-4 and ITS-6 (Cooke et al., 2000; White et al., 1990), which are universal oomycete primers. Each PCR reaction mixture was 20 µL (Table C.1).

The PCR reaction consisted of 1 cycle at 94°C for 60 seconds; 38 cycles of 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 45 seconds; and a final cycle of 72°C for 7 minutes. Successful reactions were confirmed using gel electrophoresis and products that had a clear, single band were analysed with Sanger sequencing. Sequencing results were processed using 4Peaks v 1.8 and analysed with the basic local alignment search tool (BLAST).

4.2.6 Preparation of *P. agathidicida* inoculum

Oospores were produced and isolated according to Sections 2.2.6 and 2.2.7 on three *P. agathidicida* isolate NZFS 3770 mycelial mats. Prior to inoculating soils collected from kauri in Titirangi, Tāmaki Makaurau the concentration of oospores produced was enumerated using a C-Chip™ disposable haemocytometer (DHC-N01-5, INCYTO) (Figure 4.4). According to the manufacture's manual, 10 µL sample was loaded into the chamber and oospores that were completely within the 16 squares in each of the four corners were counted at 10x magnification on an Olympus BX41 microscope. Oospores

were counted, averaged, and multiplied by a dilution factor of 10^4 for four replicate sample aliquots to yield a final concentration of approximately 2.69 x 10^5 oospores per mL.

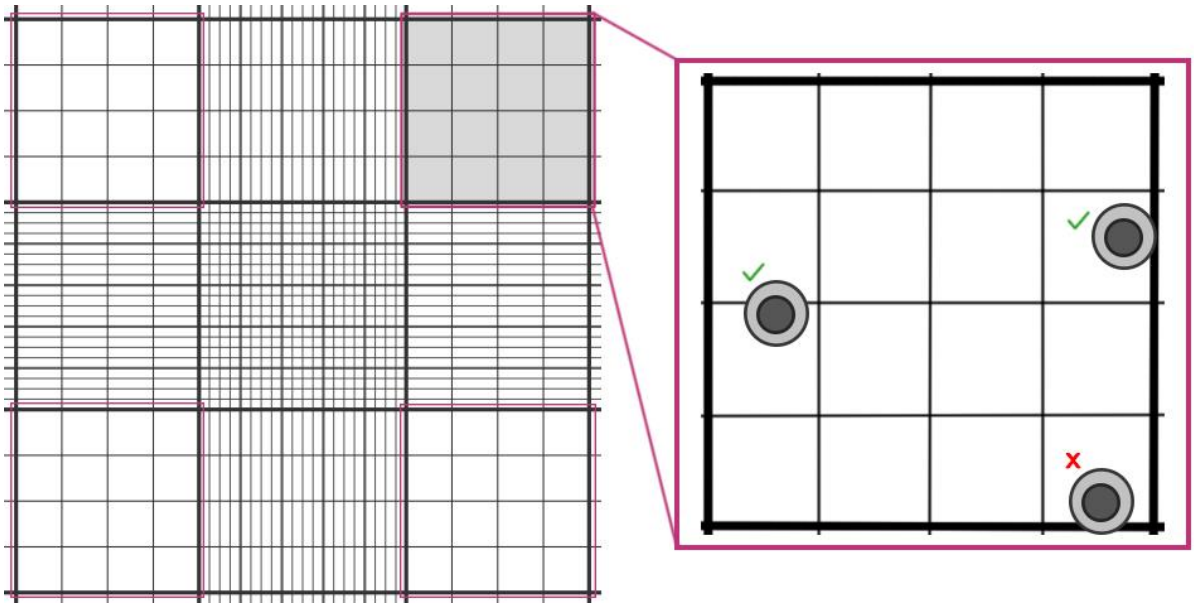

Figure 4.4: Quantifying oospores using a haemocytometer
Oospores were counted in the 16 squares in each corner, which have been outlined in pink. The close-up on the right-hand side illustrates that spores were only counted if they were completely within the borders of the square.

4.2.7 Preparation of kauri soil from Titirangi, Tāmaki Makaurau|Auckland for DNA extraction

Kauri soils were homogenised by hand and separated into 100 g subsamples. Sub-samples were either inoculated with approximately 6.72 x 10^4 *P. agathidicida* oospores or left non-inoculated as a control. Unlike Than et al. (2013), soils were freeze dried as opposed to air drying. Although there appears to be no significant difference in the community structure of extracted DNA with air drying (Clasen et al., 2020), it has been suggested that air drying soils can increase DNA degradation, and consequently, recovery (Castaño et al., 2016). Soils were transferred into 50 mL Falcon tubes that were covered with two layers of miracloth. The miracloth placement is to prevent environmental contamination while still allowing sublimation. Samples were frozen at -80°C for at least an hour, and then freeze dried (MicroModulyo, Thermo Fisher Scientific) for a minimum of four days. Samples were then stored at -20°C until extraction.

4.2.8 Extraction of DNA from soils

Soils were extracted using the methods described below. For each treatment, three replicates of *P. agathidicida* inoculated soil was used. For samples shaken by hand and those homogenised for 300 seconds using a paint shaker with 1-inch steel ball bearings, a triplicate set of controls (i.e., non-inoculated) samples were extracted as well.

4.2.8.1 Standard manual method

Extraction of DNA from soil samples was conducted based on methods previously developed by Than et al. (2013) and refined by McDougal et al. (2014).

Approximately 100 g soil was transferred to sterile, 500 mL Nalgene containers (2105-0016PK, Thermo Fisher Scientific) with ten, sterilised one-inch steel ball bearings, 200 mL 2% CTAB buffer (120 mM sodium phosphate buffer pH 8, 2% CTAB, 1.5 M NaCl), and 9 mL antifoam B emulsion (A5757, Sigma-Aldrich). Samples were shaken vigorously by hand for five minutes to mechanically lyse cells at a rate of approximately 114 ± 0.9 STE shakes per minute. Steel ball bearings were sterilised between samples by incubating them with 0.4 M HCl for 1 minute at room temperature (Qiagen, 2020), rinsing with deionized water, cleaning with 96% ethanol (Hoarau et al., 2007), and then drying at room temperature.

After shaking, 50 mL sample extract was transferred to a sterile, 50 mL tube. The sample was then centrifuged at 5,000 x ***g*** for five minutes and 20 mL clear extract was transferred to a sterile, 50 mL tube with 2 mL 5 M ammonium acetate solution. Samples were vortexed for 20 seconds to mix and then placed on ice for 10 minutes. This ammonium acetate precipitation is used to help reduce co-precipitation of contaminants such as proteins and oligosaccharides with DNA (Green & Sambrook, 2012; Singh et al., 2018).

The samples were then centrifuged at 12,000 x ***g*** for 5 minutes, after which the clear extract was transferred to a sterile, 50 mL tube containing 15 mL isopropanol and 8 mg acid washed silica sand. Nucleic acids are insoluble in isopropanol, and thus, the addition of this alcohol causes the DNA to precipitate out of solution and bind to the surface of the silica particles (Green & Sambrook, 2012; Vandeventer et al., 2013). The tubes were placed in a rack and set to shake horizontally at 70 revs/minute for 15 minutes. Tubes were then centrifuged at 12,000 x ***g*** for 5 minutes and the supernatant was discarded.

The silica pellet was re-suspended in 2 mL tissue lysis buffer (19076, Qiagen), which elutes the DNA from the silica, and set to shake horizontally at 70 revs/minute for 5 minutes at 65°C. Samples were centrifuged at 12,000 x ***g*** for 5 minutes, after which 1 mL supernatant was transferred to a sterile, 15 mL tube with 100 µL 3 M sodium acetate and 3 mL 95% ice cold ethanol. The samples were vortexed to mix and then precipitated at -20°C overnight.

The following day, the samples were centrifuged at 12,000 x ***g*** and 4°C for 30 minutes, after which the ethanol supernatant was removed via pipette. The pellets were re-suspended in 500 µL ice cold 75% ethanol, vortexed, transferred to a 2 mL tube, centrifuged at 12,000 x ***g*** and 4°C for 10 minutes, and then the supernatant wash was discarded. The pellet was washed an additional time with 500 µL ice

cold 75% ethanol, after which the tubes were set on the benchtop for 60 minutes to air dry and remove trace amounts of ethanol. The pellets were re-suspended in 100 µL 1X TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and then purified using the DNeasy® PowerClean® Pro Cleanup Kit (12997-50, Qiagen).

4.2.8.2 Automatic shaker

Shaking samples by hand has provided sufficient mechanical action to lyse and detect *P. agathidicida* in previous studies (McDougal et al., 2014; Than et al., 2013). That being said, it has been noted that shaking large soil samples by hand is laborious, and thus may vary in intensity as numerous rounds of samples are processed (McDougal et al., 2014). Some studies have eliminated this bias by using an automatic shaker, such as a paint-shaker (Reeleder et al., 2003; Woodhall et al., 2012).

In this study, soil samples were shaken using a paint shaker (S5, Santint) at a speed of 680 rpm for either 60 or 300 seconds. To prevent sample loss and reduce the possibility of contaminating the interior of the shaker, the 500 mL Nalgene bottles were fitted with a foam sleeve and re-enforced foam cap and inserted into a 4 L metal paint can.

4.2.8.3 Ball bearing size and material

DNA extraction relies on the lysis of cells, either through mechanical or chemical means. Mechanical lysis is typically induced by shaking samples with ball bearings, of which the size and material of the ball bearing can impact the quantity of DNA extracted (Reeleder et al., 2003).

While the original *P. agathidicida* manual extraction protocol calls for mechanically lysing cells with ten, one-inch steel ball bearings (Than et al., 2013), a subsequent study found that they were highly destructive to the Nalgene bottles and led to sample loss (McDougal et al., 2014). Thus, one-inch steel ball bearings were substituted with 225 g of either 4.7 mm stainless steel ball bearings or 5.0 mm glass beads. The stainless-steel ball bearings were cleaned and reused using the same protocol listed in Section 4.2.8.1 whereas the glass beads were disposed of after use.

4.2.8.4 Addition of chemical flocculants

As previously discussed in Section 4.1.1, humic acids and other organic contaminants can co-precipitate with DNA and inhibit both DNA polymerase and PCR amplification. A number of different purifying agents have been used in the manual extraction of DNA from soils to reduce co-precipitation and enhance PCR amplification (Sharma et al., 2014). Of these, the addition of 10 mmol $MgCl_2$ and 1% (w/v) activated charcoal to the extraction buffer removed PCR inhibitors most effectively, and thus, these chemical flocculants were used as a soil purifying treatment.

4.2.8.5 Use of commercial DNA extraction kits

The DNeasy® PowerSoil® and DNeasy® PowerMax® kits were used according to the manufacturer's instructions. The PowerMax® kits can process up to 10 g soil, although it is recommended that only 5 g be used in initial assays. Thus, 5 g soil was extracted using the DNeasy® PowerMax® kit.

4.2.9 Quantifying extracted DNA

Extracted DNA was quantified using a Qubit dsDNA BR Assay Kit on a Qubit 4 Fluorimeter (Invitrogen, Massachusetts USA) according to the manufacturer's instructions. Briefly, 1 µL DNA was diluted with 199 µL QuBit working solution. Samples were vortexed briefly to mix and then incubated at room temperature for 2 minutes. All samples were analysed under the broad-range function and the concentration of DNA was reported in ng/µL. Due to slight variations between absorbance readings, each sample was analysed four times and the average absorbance was reported.

4.2.10 Determining extracted DNA quality

The quality of extracted DNA was determined using a Nanodrop spectrophotometer. For each sample, the absorbance was measured for unwanted organic compounds (230 nm), DNA (260 nm), proteins (280 nm), as well as humic acids (320, 340, 350, and 465 nm) (Leite et al., 2014; Ning et al., 2009; Sharma et al., 2014; Wang & Fuiji, 2011; Yeates et al., 1998). The purity of each sample was determined by calculating the ratio of 260 nm/230 nm and 260 nm/280nm. The values for 'pure' nucleic acids were considered to be 2.0-2.2 and approximately 1.8, respectively (Laws & Adams, 1996; Lucena-Aguilar et al., 2016).

4.2.11 *Phytophthora agathidicida* detection using PCR

A PCR protocol using the *P. agathidicida* primers used by Than et al. (2013) and McDougal et al. (2014) has not been formally published. This study tested two sets of PCR reaction conditions to detect *P. agathidicida*, due to the unavailability of TaqMan™ Environmental Master Mix 2.0 required for qPCR.

DNA was amplified either using the oomycete universal primers or *P. agathidicida* specific primers (Table C.2). Amplified product generated using the universal primers were then diluted either 1:100 or 1:1000 using nuclease-free water, after which they were subjected to amplification with the *P. agathidicida* specific primers.

Two sets of PCR reaction conditions were used for the *P. agathidicida* specific primers. First, the reaction conditions listed by Than et al. (2013) were used — 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 61°C. Changes were then made to address potential issues arising from the extended initial degradation step, high annealing temperature based on primers, and the lack of an

elongation stage — 95°C for 60 seconds; 40 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes.

4.2.12 Statistical analysis

The efficacy of manual shaking methods for DNA yield and purity were analysed using one-way ANOVAs, where treatments were designated as factors. If the ANOVA showed a significant treatment effect ($p < 0.05$), a Tukey's HSD test was applied.

4.3 Results

4.3.1 Baiting kauri soil for oomycetes

A total of 33 hyphae-producing microbes were baited and isolated across the ten soil samples. Based on morphological assessments of each isolate, only 13 were selected as Sanger sequencing candidates. These isolates either had characteristics aligning with *Phytophthora* spp. (e.g., possible oospores) or had too few defining characteristics to be ruled out (e.g., no spores present) (Figure 4.5).

Sequencing of these isolates was delayed by six weeks due to restricted lab access during the Covid-19 level 4 and level 3 lockdowns. After the lockdowns, over half of the 13 sequencing candidates had lost viability and could not be successfully re-cultured. Thus, only six samples were ultimately sequenced (Table 4.3). All isolates were identified as oomycetes belonging to either the *Pythium* or *Phytophthora* genera. Of the *Phytophthora* isolates, neither was a match to *P. agathidicida*.

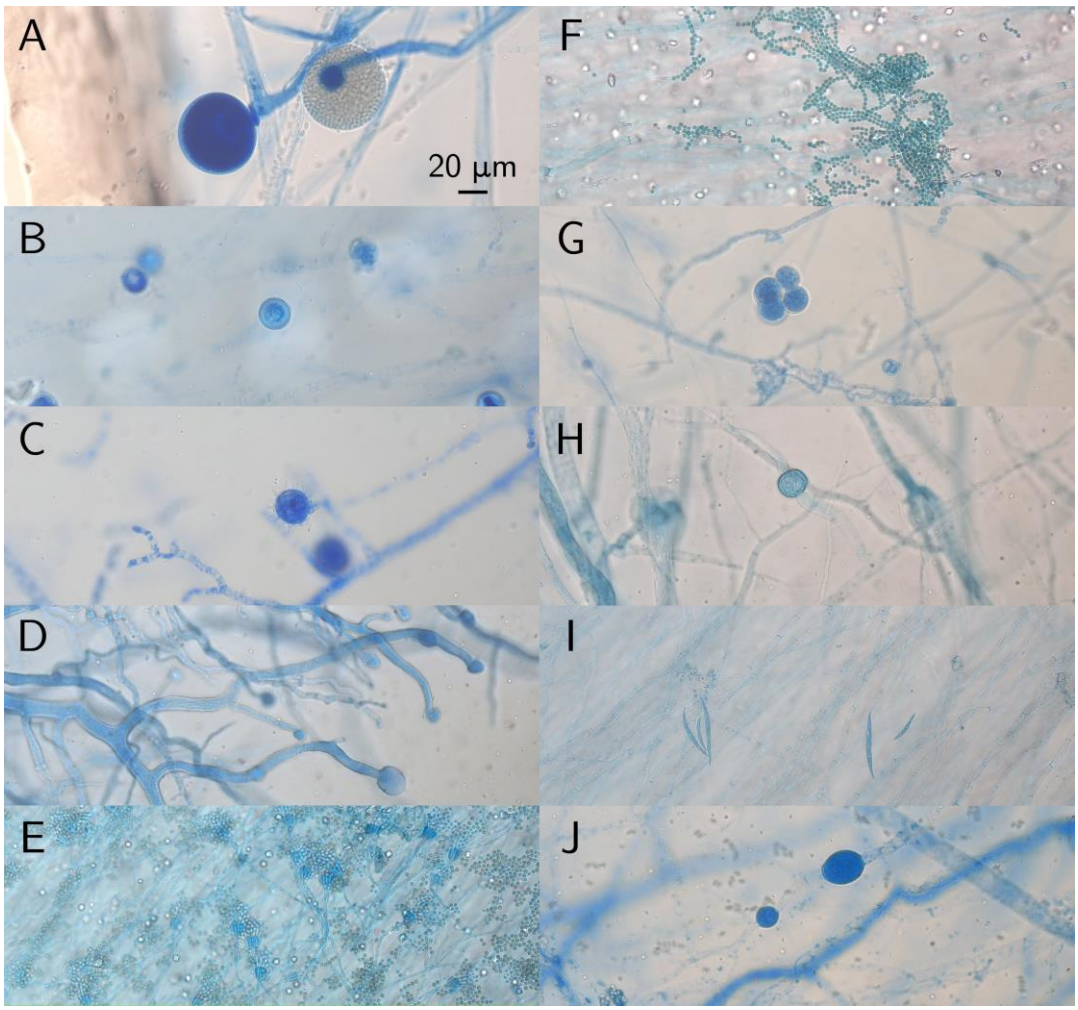

Figure 4.5. Range of spore morphology of soil baited isolates
Exact genera could not be determined based on morphological assessments alone. Rather, spores were classified as possible A| zygomycete sporangia, B| thick-walled oospore, C| *Pythium* oospore, D| juvenile spores, E| conidiophores bearing conidia, F| mature conidia, G| unidentified cluster of four, thin-walled terminal spores, H| possible zygospore or intercalary chlamydospores, I| fusiform spores, J| intercalary chlamydospores. The scale on all images is 20 µm.

Table 4.3. Identity of soil baited isolates displaying oomycota morphology as determine by Sanger sequencing and BLAST

Primer	BLAST match	Percent identity (%)
ITS-4	*Pythium* spp.	97.56
ITS-6	*Pythium attrantheridium*	98.86
ITS-4	*Pythium intermedium*	96.71
ITS-6	*Pythium intermedium*	97.21
ITS-4	*Pythium* spp.	99.94
ITS-6	*Pythium* spp.	99.86
ITS-4	*Phytophthora multivora*	99.94
ITS-6	*Phytophthora* spp.	99.73
ITS-4	*Phytophthora* spp.	99.94
ITS-6	*Phytophthora plurivora*	100
ITS-4	*Pythium* spp.	99.81
ITS-6	*Pythium* spp.	99.87

4.3.2 Eliminating sample loss with shaking

Nalgene bottles were shaken with a range of foam padding levels within the 4 L paint can, with the aim of reducing sample loss. Sample loss only occurred through damage to the cap, although this was eliminated by fitting the cap with a foam cover that was reinforced with a hard plastic insert (Figure 4.6).

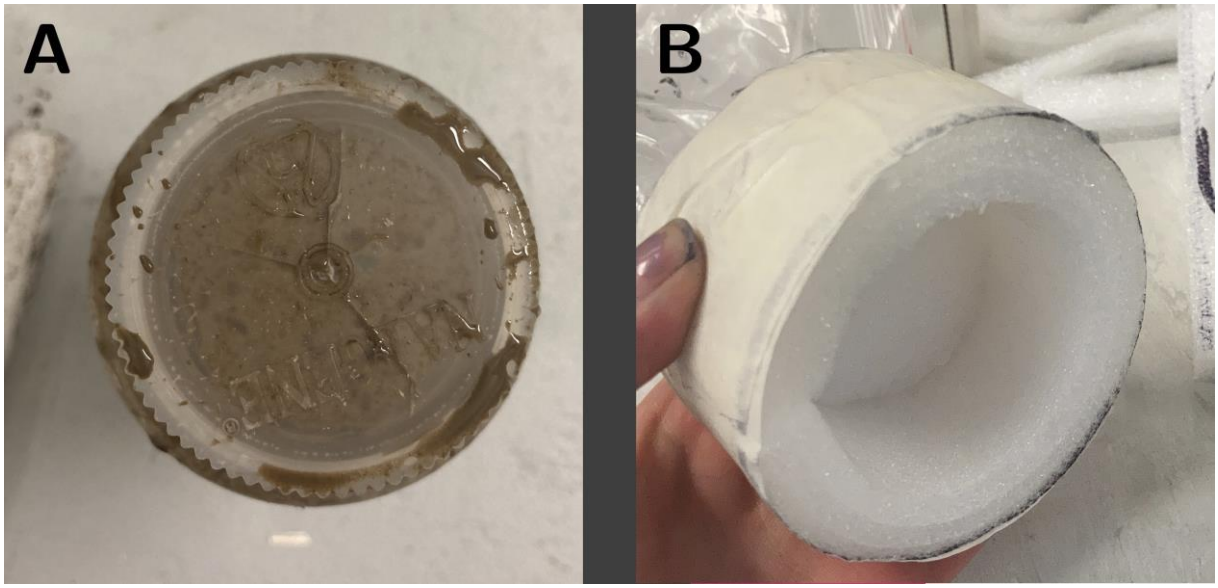

Figure 4.6: Damage to 500 mL Nalgene caps using 1-inch steel ball bearings
A| Cracked lid and sample loss after shaking with a paint shaker for 60 seconds. B| foam cap with plastic insert that prevented sample loss with shaking.

4.3.3 Optimisation of DNA yield from soils using manual extraction methods

The average yield of DNA extracted using manual methods increased with the use of a paint shaker (Figure 4.7). Of the paint shaker extraction treatments, shaking for 60 seconds with one-inch steel ball bearings had the lowest DNA yield, although it was not statistically different than that of shaking for five minutes with either one-inch steel or 4.7 mm stainless steel ball bearings. Based on the applied treatments, using 5 mm glass beads significantly improved DNA extraction yield when compared to shaking by hand.

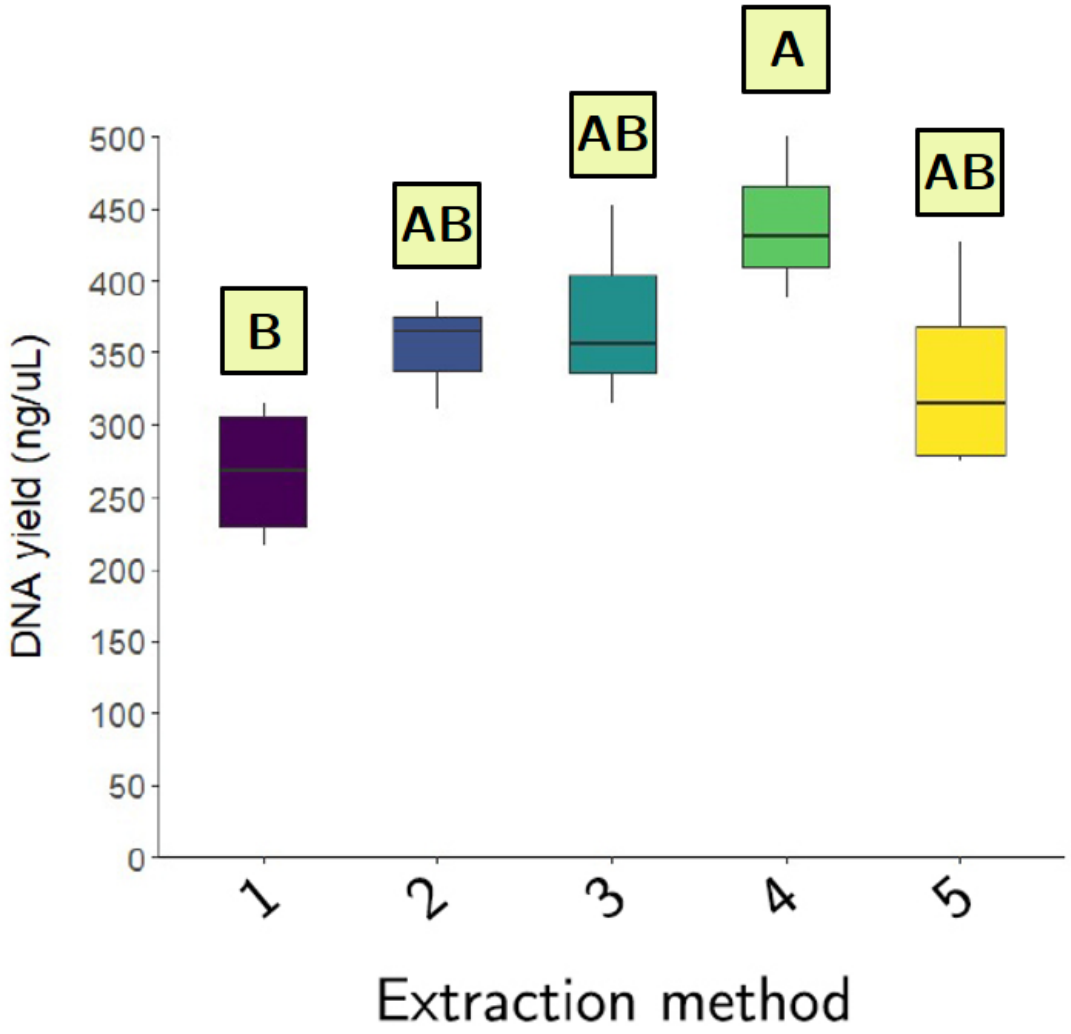

Figure 4.7: Difference in extracted DNA yield (ng/µL) between different shaking methods and the use of ball bearings of different sizes and materials
The extraction methods used were 1| hand shaking for five minutes using 1-inch steel ball bearings, 2| automatic shaker for five minutes using 1-inch steel ball bearings, 3| automatic shaker for five minutes using 4.7 mm stainless steel ball bearings, 4| automatic shaker for five minutes using 5.0 mm glass beads, and 5| automatic shaker for one minutes using 1-inch steel ball bearings. The error bars shown depict the 95% confidence interval based on three replicates. Yield values that share the same letter across different treatments (e.g., 1, 2, etc.) are not statistically different ($p < 0.05$, Tukey's test).

When analysing extracted DNA purity, no absorbance was registered between 340 and 465 nm for any sample. DNA that had been extracted without the use of chemical flocculants or the DNeasy® PowerClean® Pro Cleanup Kit was contaminated with proteins, humic acids, and other organic contaminants (Error! No bookmark name given.**). Adding $MgCl_2$ and activated charcoal as chemical flocculants had no effect on absorbance at 320 nm, but significantly affected the 260/230 ratios, with a p-value of 4.33×10^{-2}. This suggests that the use of chemical flocculants did significantly improve humic acid contamination, however, the samples were still too contaminated to be used for PCR without further purification. On the other hand, the DNeasy® PowerClean® Pro Cleanup Kit consistently yielded 260/280 ratios of approximately 1.8 and 260/230 ratios of between 2.0 and 2.2.**

Table 4.4: Purity of extracted DNA as determined by spectrophotometry

Treatment		320 nm absorbance	260/280 absorbance ratio	260/230 absorbance ratio
Hand shaking		$1.30 \times 10^{-2} \pm 1.54 \times 10^{-3}$	$1.85 \pm 1.00 \times 10^{-2}$	$2.06 \pm 1.26 \times 10^{-2}$
Paint shaker				
One-inch steel ball bearings	60s	$2.07 \times 10^{-2} \pm 4.62 \times 10^{-3}$	$1.85 \pm 2.97 \times 10^{-3}$	$2.15 \pm 2.13 \times 10^{-2}$
	300s	$2.97 \times 10^{-2} \pm 8.30 \times 10^{-3}$	$1.83 \pm 1.91 \times 10^{-3}$	$2.09 \pm 4.83 \times 10^{-2}$
4.7mm stainless steel ball bearings	300s	$4.03 \times 10^{-2} \pm 1.54 \times 10^{-2}$	$1.84 \pm 9.18 \times 10^{-2}$	$2.08 \pm 4.46 \times 10^{-2}$
5.0mm glass ball bearings	300s			
Chemical flocculants				
Control		3.93 ± 0.56	$1.68 \pm 1.53 \times 10^{-3}$	$0.37 \pm 1.59 \times 10^{-2}$
$MgCl_2$ + activated charcoal		4.99 ± 0.28	$1.63 \pm 4.58 \times 10^{-3}$	$0.53 \pm 3.95 \times 10^{-2}$

4.3.4 Detection of *P. agathidicida* using different extraction methods

Amplification of DNA extracts using only the *P. agathidicida* specific primers showed signs of non-specific binding and low amplification of the desired product (Figure 4.8a). Non-specific binding was not observed in the nested PCR, although the band strength was faint. Adjusting the parameters to optimise the PCR thermal cycling conditions did not yield improved band appearance or strength.

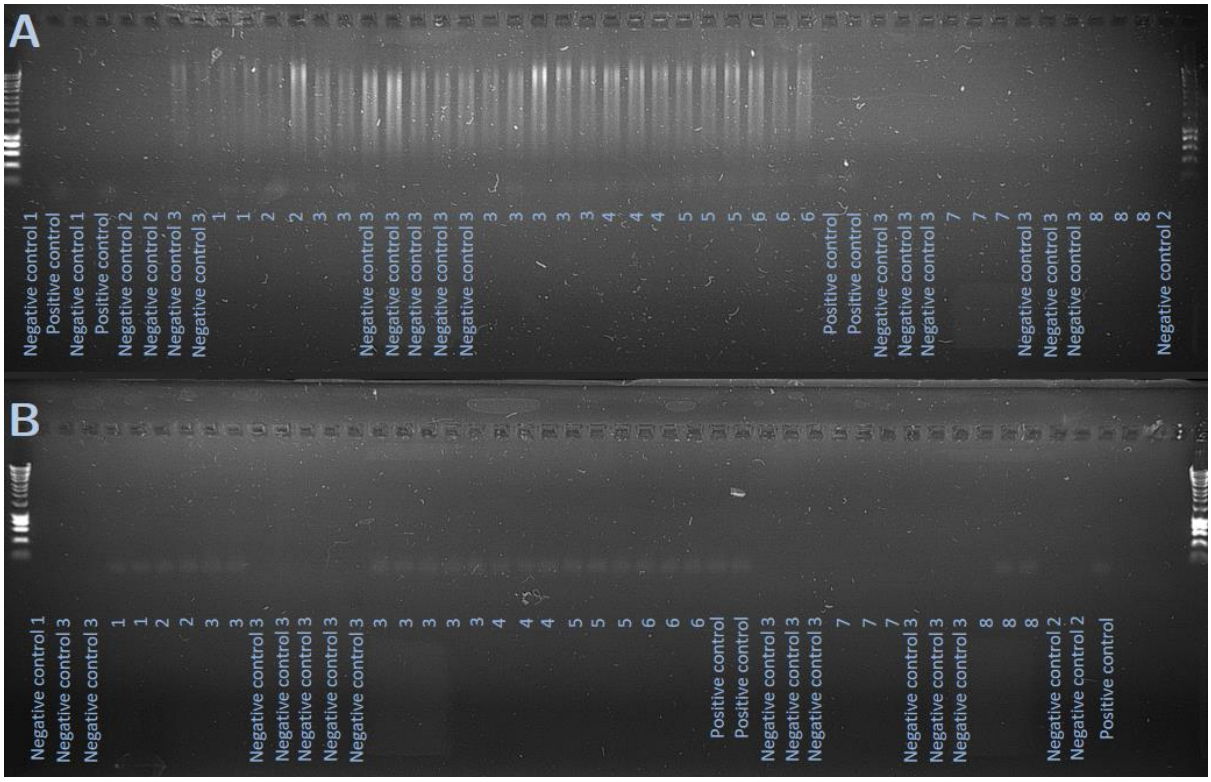

Figure 4.8: Gel electrophoresis of PCR products using *Phytophthora agathidicida* specific primers and universal oomycete primers
A| PCR product using *P. agathidicida* specific primers. B| Nested PCR product using universal oomycete primers followed by *P. agathidicida* primers. Beneath each lane is an indication of the type of control or treatment applied. Negative controls 1 and 2 refer to a PCR water blank and a non-*P. agathidicida* oomycete respectively. Negative control 3 refers to extracted soil that had not been inoculated with *P. agathidicida*. The extraction treatments applied were 1| handshaking for 5 minutes using 1-inch steel ball bearings, 2| Automatic shaker for 1 minute using 1-inch steel ball bearings, 3| Automatic shaker for 5 minutes using 1-inch steel ball bearings, 4| Automatic shaker for 5 minutes using 4.7 mm stainless steel ball bearings, 5| Automatic shaker for 5 minutes using 5.0 mm glass ball bearings, 6| Automatic shaker for 5 minutes using 1-inch steel ball bearings and chemical flocculants, 7| PowerSoil® kit, and 8| PowerMax® kit.

Phytophthora agathidicida was not detected in any of the non-inoculated soils, which suggests that the Titirangi site is still likely free from kauri dieback. The pathogen was detected in all inoculated soils that were extracted manually, regardless of shaking apparatus, ball bearing size or material, or the chemicals used in the extraction buffer. *P. agathidicida* was less consistently detected using the commercial DNA extraction kits — only two of the three PowerMax® and none of the PowerSoil® replicates detected the pathogen.

4.4 Discussion

4.4.1 Persistent issues with soil baiting

The issues that have been previously associated with soil baiting — specifically, unreliability of culturing *Phytophthora*, difficulties conducting accurate morphological assessments, and assay cost — were confirmed by this assay.

Even on media designed to enhance *Phytophthora* spp. isolation, microbial colonies were frequently produced from bait tissue (Figure 4.2). This indicates that the media designed for *P. agathidicida* growth is not selective enough to reliably isolate oomycetes. It is possible that this is an artefact of substituting PCNB for nystatin, although regardless of its cause, this indicates that the selective media formula for *P. agathidicida* isolation could be further optimised.

Furthermore, the two *Phytophthora* isolates baited by this study were entirely devoid of spores and consequently could not be identified by morphology alone. This meant that all possible *Phytophthora* isolates required the use of an extraction kit and Sanger sequencing to identify.

Hybrid detection methods for *P. agathidicida* have previously utilised both DNeasy® UltraClean® Microbial and the Macherey-Nagel™ NucleoSpin™ Plant II kits (Winkworth et al., 2020; A-K. Byers, Scion, Personal communication), which cost approximately $4 and $4.8 NZD per sample respectively. Using the DNeasy® UltraClean® Microbial kit with the extended soil bioassay offers a few potential benefits. The kit itself is less expensive and assay costs can be further reduced by eliminating samples with morphologies inconsistent with *Phytophthora*. Also, as this method extracts pure isolates, it can be compatible with the universal oomycete primers, which can alert land managers to the presence of other, pathogenic oomycete species. On the other hand, while extracting the baits directly would have at least doubled extraction kit cost in this study, it would have reduced overall assay time by approximately ten days, eliminated the need for many consumables (e.g., Petri dishes), and would likely have yielded a more accurate *P. agathidicida* detection rate if the pathogen was present (Winkworth et al., 2020).

4.4.2 Optimising manual extraction methods for *P. agathidicida*

The yield of extracted DNA from manual methods was improved by adjusting the parameters for cell lysis. Glass ball bearings produced the highest average DNA yield, although this finding should be confirmed with additional replication. There was relatively little difference between DNA yield using steel ball bearings of varying sizes, however both sizes presented certain cross contamination risks. The 1-inch steel ball bearings presented with visible surface imperfections after use, which risks carrying over DNA between samples. The 4.7 mm stainless steel ball bearings were frequently

aggregated in the clay soil, and thus would likely have benefitted from a ten-minute soak in 10% bleach or other more intensive wash method. On the other hand, the glass ball bearings remained intact and without visible flaws. Thus, it would be worth considering if glass ball bearings could be sterilised between uses, rather than disposed.

While DNA yield garnered from manual extraction methods was improved, a commercial clean-up kit is still required to remove protein and humic acid contamination prior to PCR amplification. Even with the added cost of the DNeasy® PowerClean® Pro kit, the cost per sample was only twice as expensive as the DNeasy® PowerSoil® and much more effective at detecting *P. agathidicida* (Table 4.5).

Table 4.5: Comparison of cost, sample size, processing time, and additional consumables and equipment for different soil extraction methods

	Manual extraction	DNeasy® PowerSoil®	DNeasy® PowerMax®
Cost per extraction ($NZD)	20.78[1]	9.2	40
Soil sample size (g)	100	0.25	10
Approximate processing time for 24 samples (h)	6.5[2]	2.5	5.5
Additional consumables	Nalgene bottles Ball bearings		
Additional required equipment	Paint shaker 2500 x ***g*** centrifuge with 50 mL adaptors Pipettors (50-1000 µL) Vortex Genie® 2 Horizontal shaker (70 revs/min)	Microcentrifuge Pipettors (50-1000 µL) Vortex Genie® 2 Vortex adaptor for 24 samples	2500 x ***g*** centrifuge with 50 mL adaptors Pipettors (1 and 10 mL) Vortex Genie® 2 Vortex adaptor for 2x 50 mL tubes

[1] Includes cost of DNeasy® PowerClean® Pro kit

[2] Not including overnight precipitation

There are additional changes that could be applied to the manual extraction method to possibly increase extract purity. Precipitation with polyethylene glycol (PEG), as opposed to ethanol, has been used to remove humic acids (Biver & Vandenbol, 2013). A phenol:chloroform extraction can also be added prior to DNA precipitation to remove proteins (Sharma et al., 2014; Wilson, 1997), although phenol itself is highly toxic (Chacon-Cortes & Griffiths, 2014; Martin et al., 2012). Alternatively, proteins could be further removed through a proteinase K digestion (Fredricks et al., 2005; Laws & Adams, 1996).

4.4.3 Issues surrounding relic DNA

One benefit of soil baiting is that it exclusively detects infectious, viable pathogen. Molecular methods, on the other hand, do not discriminate between viable and relic, or extracellular, DNA. Extracellular DNA — from non-viable, lysed cells — can persist for years in soil (Carini et al., 2016). The longevity of relic DNA depends in part on the soil type, as soils with clay minerals and humic acids adsorb nucleic acids, which protects it from degradation (Levy-Booth et al., 2007; Ogram et al., 1988). Based on the composition of kauri soils, it is likely that relic DNA would be a non-negligible component of total nucleic acids extracted.

Soil samples can be treated with a viability dye, such as propidium monoazide (PMA), which bind to extracellular DNA. The bound PMA is then activated by certain light conditions, which permanently damages the extracellular DNA and prevents its amplification (Emerson et al., 2017). Studies that have treated soil with PMA have found that relic DNA can account for between 14 and 40% of DNA present (Carini et al., 2016; Wagner et al., 2015).

If molecular methods are to replace soil baiting or hybrid methods as the dominant method for kauri dieback detection from environmental samples, it would be pertinent to incorporate PMA treatment into the SOP, as this would provide those managing kauri dieback with a more accurate representation of current disease presence and threat.

4.4.4 Optimising PCR conditions for *P. agathidicida* specific primers

Despite changes to the thermal cycling sequence for *P. agathidicida* amplification, gel electrophoresis showed high molecular weight streaking on non-nested PCR products and low amplification of the desired product in the nested PCR (Figure 4.9).

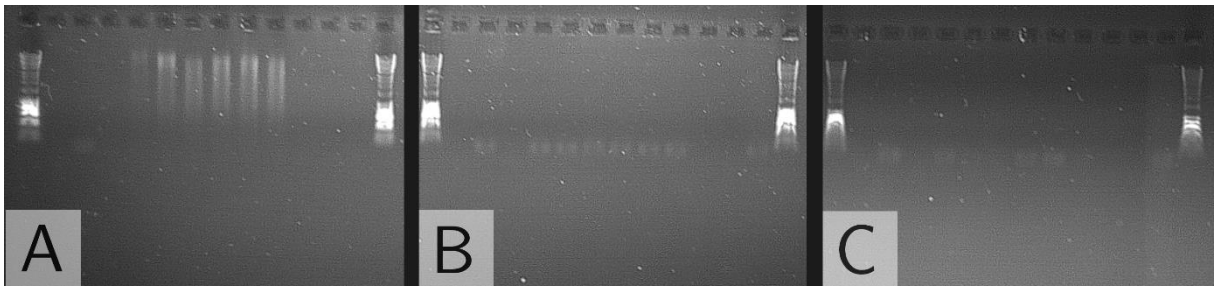

Figure 4.9: Gel electrophoresis of PCR products using an updated thermocycling scheme that reduced initial denaturation period, lowed the annealing temperature, and added an elongation step
A| *P. agathidicida* specific primers. B| Nested PCR using 1:100 diluted product. C| Nested PCR using 1:1000 diluted product.

The persistent streaking using only the *P. agathidicida* specific primers could indicates nonspecific amplification. While the exact cause of this could not be elucidated without further experimentation, some possible causes are excessive concentration of template DNA, which could be remedied by

diluting extracted DNA, or the high number of thermal cycles, which could be reduce from 40 cycles to 35 or fewer.

Regarding the low product yield, it is possible that the initial denaturation period is too short, which could be increased from 1 minute to 3 minutes. Alternatively, the annealing temperature may still be too high, and could be reduced from 57°C to 52°C.

4.4.5 Possible application of 3rd generation sequencers

Third-generation sequencing (TGS) refers to a group of DNA sequencing methods and instruments that are actively being developed and offer an exciting range of possibilities for pathogen detection. These technologies can potentially decrease sequencing time (Loit et al., 2019), reduce the cost per run (Boykin et al., 2019), and even be used to process samples in the field (Boykin et al., 2019; Goordial et al., 2017; Parker et al., 2017; Pomerantz et al., 2018).

The development of a TGS method for *P. agathidicida* detection would greatly benefit kauri health and dieback outcomes. The low cost per run could allow for proactive sampling schemes focused on early detection. This contrasts with current testing, where samples are generally taken as a means of disease confirmation after physical symptoms have been expressed (Winkworth et al., 2020). Furthermore, TGS devices are considered to be less complex and may enable a wider population to become involved in disease management. Therefore, this technology could act to empower mana whenua iwi, many of which consider the health of kauri to be their right and responsibility (Nuttall et al., 2010).

4.5 Conclusion

This study found that changes made to the manual *P. agathidicida* extraction method for soil samples reduced sample loss and increased DNA yield. Additional work still needs to be done to improve DNA extract purity, which would avoid the need for a commercial clean-up kit and further reduce assay cost.

Commercial DNA extraction kits, on the other hand, were found to be less reliable in *P. agathidicida* detection, although this could potentially be countered through pooling multiple sub-samples. It is unclear, however, as to the number of subsamples needed to achieve comparable detection rates.

Pressing next steps in this line of work include comparing the detection thresholds between these methods (manual and commercial) using quantitative PCR as well as continuing to optimise the thermal cycling conditions for PCR to improve product yield.

Chapter 5

Conclusion

5.1 Summary and key results

This study aimed to improve methods for kauri dieback prevention and *P. agathidicida* detection in environmental samples. In pursuit of these goals, methods to study oospore viability were also compared and improved upon.

The main objectives of this thesis were to:

1. Compare the efficacy of methods to isolate *P. agathidicida* oospores and determine their viability.

2. Evaluate the effect of four fungicides (ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin) and five essential oils (*T. vulgaris*, *M. alternifolia*, *P. graveolens*, mānuka (*L. scoparium*), and kānuka (*K. ericoides*)) on the viability of *P. agathidicida* mycelia *in vitro*, as well as identify further anti-*Phytophthora* compounds within the tested essential oils.

3. Optimise the manual extraction of *P. agathidicida* DNA from soil samples.

5.1.1 Conducting assays with *P. agathidicida* oospores

Oospores were isolated most readily with the use of digestive enzymes from *T. harzianum*, which minimised the mycelial fragmentation and contamination previously documented (Williams, 2015). This appears to be the first study to use digestive enzymes to isolate *P. agathidicida* oospores, although it has been successfully applied with other oomycetes (Lees et al., 2012; Porter et al., 2007).

Oospore viability methods could not be compared due to low germination rates on media coupled with issues plasmolysising oospores. However, MTT vital staining did successfully differentiate viable spores and accurately reflected a decline in spore viability when exposed to increasing heat, similar to other *P. agathidicida* studies (Dick & Kimberley, 2013; Williams, 2015). Oospore viability with MTT vital staining was assessed both visually and with the use of Streams, an image processing software designed for particle detection, the latter of which was a novel application. Streams had an 86% agreement with visual based assessments, only had a 1% false positive rate, and was able to processing 150 images containing over 450 spores in merely 35 seconds, a fraction of the time taken to visually assess the images.

5.1.2 Possibility for new kauri dieback preventative tools

Ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin were found to inhibit *P. agathidicida* mycelial growth at very low concentrations (EC_{50} values ranging from 1.70×10^{-4} to 3.89×10^{-1} µg/mL). Oxathiapiprolin was found to be the most effective inhibitor of *P. agathidicida* mycelia, both in the selection of fungicides used in this experiment as well as the entirety of antimicrobial compounds previously screened by other studies (Table 3.8).

The essential oils screened in this study were less effective at inhibiting *P. agathidicida* mycelia (EC_{50} values ranging from 7.88×10^{-2} to 3.28 µL/mL). Interestingly, both indigenous plant essential oils produced the lowest mycelial inhibition rates, although this is likely due to the fact that these products either lacked or had low concentrations of potent β-triketones and flavonoids previously identified in extracts from these plants (Lawrence et al., 2019; Park et al., 2017; van Klink et al., 2005). Of the compounds identified in the five essential oils, approximately 16% have previously demonstrated antimicrobial properties and approximately 3% are known to inhibit other *Phytophthora* pathogens.

5.1.3 Improved detection of *P. agathidicida* from environmental samples

DNA yield from manually extracted soil samples was increased by eliminating catastrophic sample loss, as a consequence of damaged Nalgene vessels, and using an automatic shaker, as opposed to hand shaking. Efforts to reduce protein and humic acid contamination from extracts were not successful, and thus, DNA extracts still require the use of a commercial clean-up kit for downstream analyses.

Phytophthora agathidicida was detected from all manually extracted samples — regardless of shaking method or ball bearing size or material — but inconsistently detected using commercial DNA extraction kits. As the commercial DNA extraction kits share the same chemistry, this indicates that the increased detection rate using the DNeasy® PowerMax® kits (2/3 samples) compared to DNeasy® PowerSoil® kits (0/3 samples) is related to differences in the quantity of soil extracted, wherein a greater mass of soil is required for accurate *P. agathidicida* detection.

5.2 Future directions

5.2.1 Further development of image processing software for cell viability assays

This study highlighted the potential to use Streams, an image processing software, to determine cell viability. Streams is both time and cost effective and operates without systemic bias. To further validate its accuracy, a greater number of visual assessments using vital stains are needed from additional, trained technicians. This will act to establish a more accurate positive and false detection

rate using Streams. With the presence of blue spores observed in Section 3.3.9, an additional filter pipeline sequence that is optimised to isolate and amplify this colour range is also needed.

5.2.2 Understanding the effect of anti-*Phytophthora* fungicides on gene expression

When exposed to increasing concentrations of certain fungicides, *P. agathidicida* presented with mycelial morphological changes (Figure 3.5). This could indicate that the fungicides, namely fluopicolide and oxathiapiprolin, are causing visible changes in gene expression. Alternatively, some of these fungicides are known to affect the structural formational of oomycete cells (i.e., microtubule formation), which could cause visible morphologicial changes in the appearance of the culture. These changes could be characterised through the use of RNA sequencing and transcriptome analysis, which may provide valuable insight into the modes of action of these fungicides as well as pinpoint vulnerable biological pathways in *P. agathidicida*.

5.2.3 Determining the effect of anti-*Phytophthora* fungicides on *P. agathidicida* sporangia, zoospores, and oospores

While this study determined the EC_{50} values for ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin on *P. agathidicida* mycelia, a dominant structure in infected root systems, further work is needed to understand how they affect the viability of oospores, the dormant survival spore, and both sporangia and zoospores, which are asexual reproductive spores that contribute significantly to new infections. Understanding how these fungicides effect other *P. agathidicida* spores will act to inform treatment management (e.g., application of fungicides as soil drenches, use in boot-wash stations, etc.).

Earlier this year, a pre-print reported the inhibition (EC_{50} values) of oxathiapiprolin on *P. agathidicida* mycelial growth and the germination of both oospores and zoospores (Lacey et al., 2021a). It would be beneficial to apply the methods in this pre-print on a wider range of both *P. agathidicida* isolates, to account for geographical variation (Winkworth et al., 2021), and fungicides, like ethaboxam, fluopicolide, and mandipropamid.

5.2.4 Determining the specificity of anti-*Phytophthora* fungicides

One concern regarding the use of fungicides in indigenous landscapes is that they may have low specificity, which could harm the overall soil microbial community and their functions. Prior to field applications, appropriately concentrated solutions of each fungicide should be applied to soil samples taken from multiple locations within kauri forests. The soil microbial community structure and function between treated and non-treated soils can then be compared, the methods for which have been established by a previous study (Byers et al., 2020).

These fungicides are also noted for their potential toxicology, especially with relation to aquatic organisms. Thus, it may be necessary to determine the environmental persistence of these fungicides as well as study the hydrological patterns and drainage of groundwater in kauri dieback locations.

5.2.5 Applying fungicides to kauri

Additional research is needed to understand the interaction between the four fungicides used in this study with diseased kauri. A glasshouse trial modelled after Horner & Hough (2013) would allow for the determination of whether commercial formulations of these fungicides could be effectively applied as either a lesion treatment or soil dredge and whether these fungicides possess preventative or curative properties. When conducting these trials, it would also be beneficial to assess the effect of mixtures combining multiple anti-*Phytophthora* fungicides, as fungicides may act synergistically to provide a larger treatment effect (Gisi et al., 1985; Grabski & Gisi, 1987; Samoucha & Cohen, 1986; Wang et al., 2014).

5.2.6 Conducting a *P. agathidicida* recovery assay using qPCR

Due to time and material constrains caused by Covid-19, this study was only able to compare *P. agathidicida* detection rates between different extraction methods using PCR. In order to recommend the optimised manual DNA extraction method over soil baiting, it is critical to know the detection threshold of each method, which can be established with real-time PCR (qPCR).

Future detection threshold studies should utilise multiple known concentrations of *P. agathidicida* oospores, possibly based off those used by Than et al. (2013). While this study used three replicates for each treatment, it is recommended than additional replication (e.g., 10 replicates) be used for qPCR to provide greater confidence in the significance of treatment effect. It would be beneficial if future detection threshold studies compared at least the following methods: soil baiting, the optimised manual DNA extraction protocol, and both DNeasy® PowerSoil® and PowerMax® kits.

5.2.7 Purifying soil DNA extracts

The use of chemical flocculants in DNA extraction buffer did not adequately reduce protein and humic acid contamination of DNA. Thus, the methods used in this study still require the use of a commercial clean-up kit prior to PCR, which adds an additional $5 NZD cost to every sample. To further reduce samples cost, alternative chemicals and methods can be tested. For example, proteinase K digestions can be used to remove proteins, precipitation can be conducted with polyethylene glycol (PEG), rather than ethanol, or a phenol:chloroform extraction can be used before precipitating DNA (Biver & Vandenbol, 2013; Fredricks et al., 2005; Laws & Adams, 1996; Sharma et al., 2014; Wilson, 1997).

5.3 Conclusion

Phytophthora agathidicida threatens the longevity of the culturally and ecologically important kauri. Despite current available treatments and efforts to characterise the range and spread of *P. agathidicida*, more effective tools are needed on both the prevention and detection front. This thesis successfully built on prior studies to add to our knowledge regarding molecular detection of kauri dieback in soils and identification of new inhibitors of the pathogen that causes it. While these are promising developments, follow-up research is needed to compare the detection thresholds between the current soil detection SOP and the modified manual extraction methods presented here, determine the effect of fungicides and antimicrobial compounds identified from plant essential oils across various *P. agathidicida* life cycle stages, and understand the effect of fungicide treatments on the wider indigenous forest landscape. By further pursuing these objectives, it will provide land managers with additional strategies and techniques that are critical for the long-term survival of kauri.

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Appendix A

Supplemental data for Chapter 2

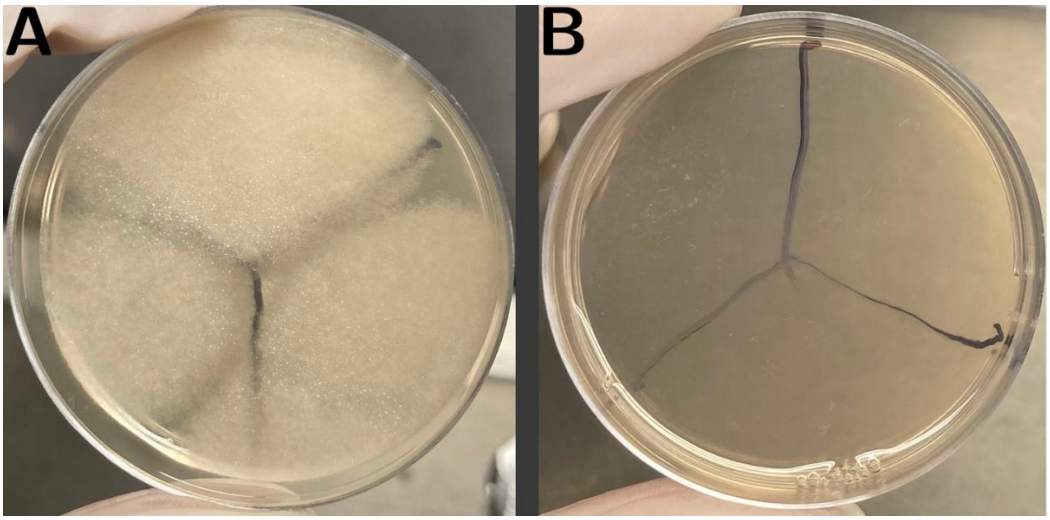

Figure A.1: *Phytophthora agathidicida* oospores germinated on selective agar media
A| Clear germination of oospores heat-treated at room temperature (22°C) after a one-week incubation.
B| Lack of germination and mycelial production of oospores heat-treated at 50°C after a four-week incubation.

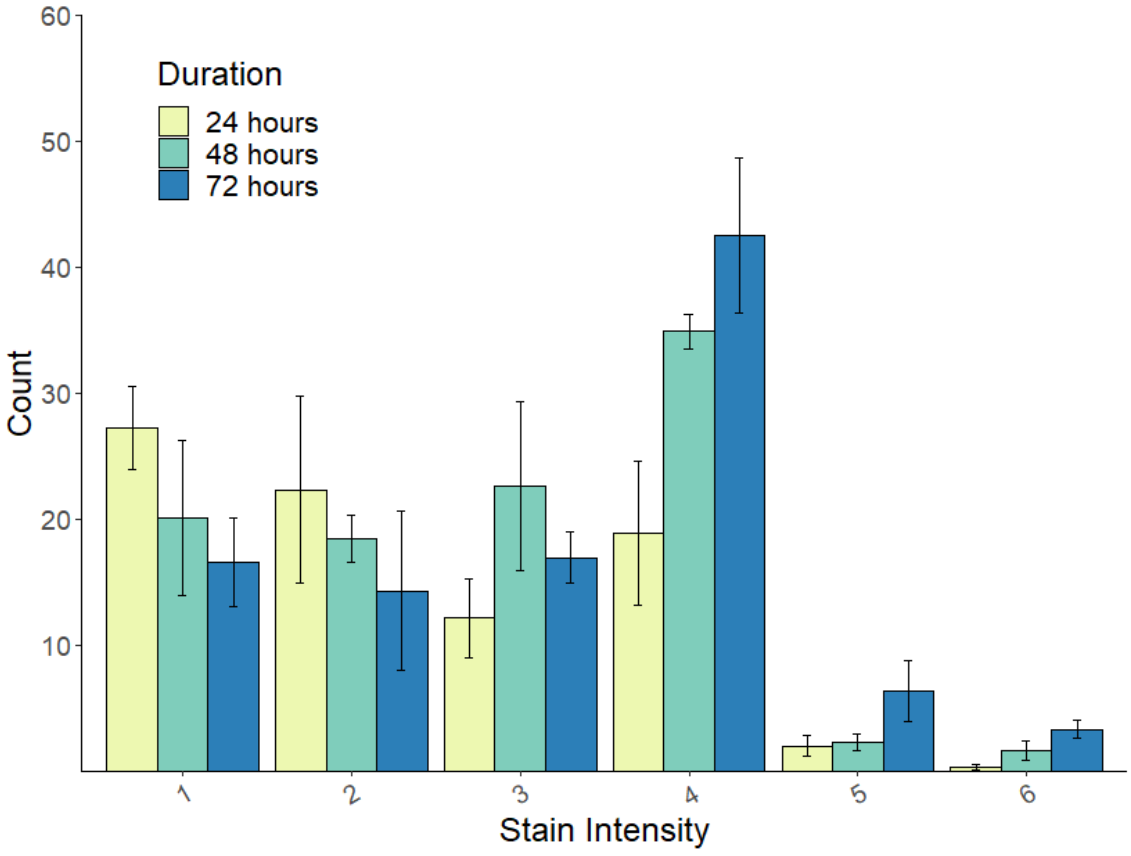

Figure A.2: Intensity of thiazolyl tetrazolium bromide staining in *Phytophthora agathidicida* oospores after 24, 48, and 72 hours of exposure to the vital stain
The degrees of intensity are classified as 1| clear, 2| pale pink, 3| pink, 4| purple, 5| dark purple, and 6| black. Error bars depict the standard error based on three replicates.

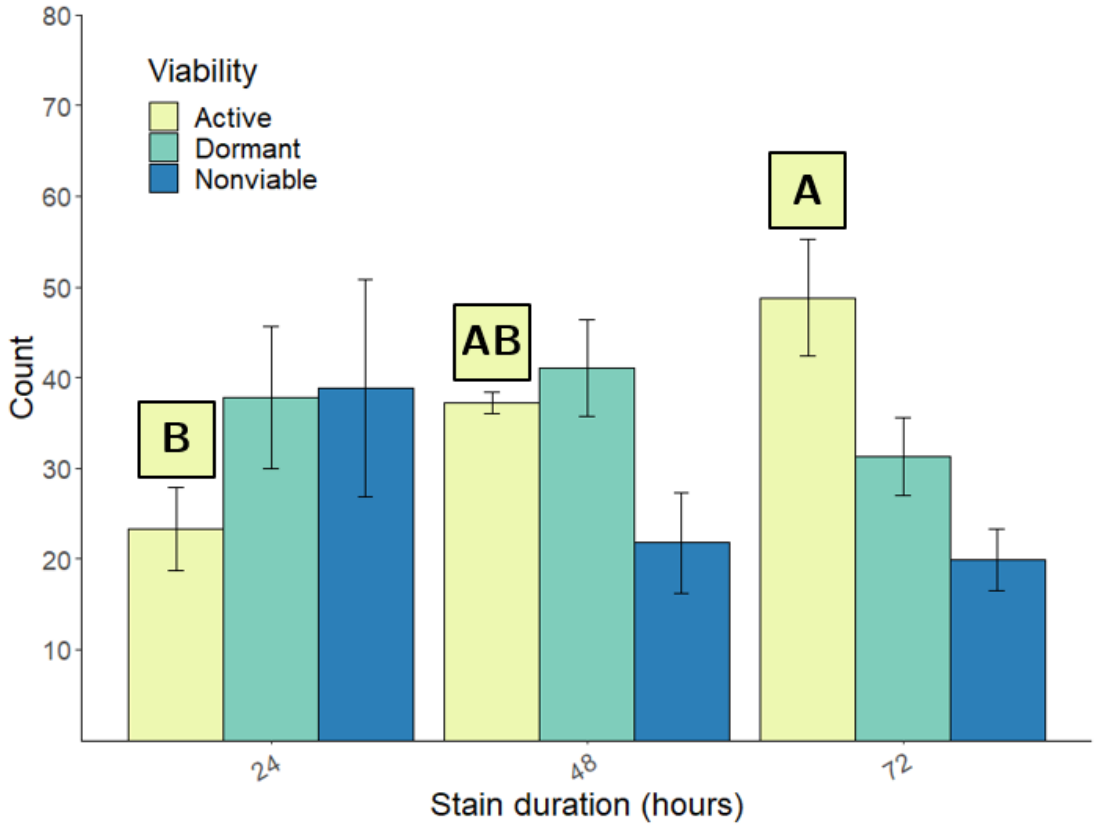

Figure A.3: MTT viability assessment of *Phytophthora agathidicida* oospores after 24, 48, and 72 hours of exposure to thiazolyl tetrazolium bromide

Error bars depict the standard error based on three replicates. Viability counts that share the same letter within the same category (e.g., active, dormant, etc.) across different incubation periods (e.g., 24, 48, 72 hours) are not statistically different ($p < 0.05$, Tukey's HSD test).

Table A.1: Post hoc comparisons of the number of hyphal fragments produced by different methods of oospore isolation using the Tukey HSD test

The different isolation methods were homogenisation (H), sonication (S), enzyme digestion (E), homogenisation followed by sonication (HS), homogenisation followed by enzyme digestion (HE), sonication followed by enzyme digestion (SE), and homogenisation followed by sonication followed by enzyme digestion (HSE).

Contrast	Difference of Means	Adjusted p-Value
S-H	-2.9326867	0.0001002
E-H	-0.5489806	0.6110364
HS-H	1.8891947	0.0035832
HE-H	-1.0955483	0.0994476
SE-H	-0.9135748	0.1480497
HSE-H	-0.5351366	0.6353779
E-S	2.3837061	0.0002723
HS-S	4.8218814	0.000001
HE-S	1.8371384	0.0043978
SE-S	2.0191119	0.0010665
HSE-S	2.3975502	0.0002593
HS-E	2.4381753	0.0002248
HE-E	-0.5465678	0.6152775
SE-E	-0.3645942	0.8429109
HSE-E	0.013844	1
HE-HS	-2.984743	0.0000859
SE-HS	-2.8027695	0.000067
HSE-HS	-2.4243313	0.0002359
SE-HE	0.1819735	0.9962567
HSE-HE	0.5604118	0.590976
HSE-SE	0.3784382	0.8208827

Table A.2: Post hoc comparisons of the number of intact oospores produced by different methods of oospore isolation using the Tukey HSD test

The different isolation methods were homogenisation (H), sonication (S), enzyme digestion (E), homogenisation followed by sonication (HS), homogenisation followed by enzyme digestion (HE), sonication followed by enzyme digestion (SE), and homogenisation followed by sonication followed by enzyme digestion (HSE).

Contrast	Difference of Means	Adjusted p-Value
S-H	-4.5	0.9992918
E-H	45.791667	0.009629
HS-H	5.375	0.9980926
HE-H	24	0.3416692
SE-H	25.958333	0.1962521
HSE-H	21.541667	0.3580518
E-S	50.291667	0.0049977
HS-S	9.875	0.957845
HE-S	28.5	0.1946062
SE-S	30.458333	0.1004678
HSE-S	26.041667	0.1939107
HS-E	-40.416667	0.0216481
HE-E	-21.791667	0.3468417
SE-E	-19.833333	0.3301585
HSE-E	-24.25	0.1659328
HE-HS	18.625	0.5968846
SE-HS	20.583333	0.4032367
HSE-HS	16.166667	0.6449907
SE-HE	1.958333	0.9999904
HSE-HE	-2.458333	0.9999635
HSE-SE	-4.416667	0.9980247

Appendix B

Supplemental data for Chapter 3

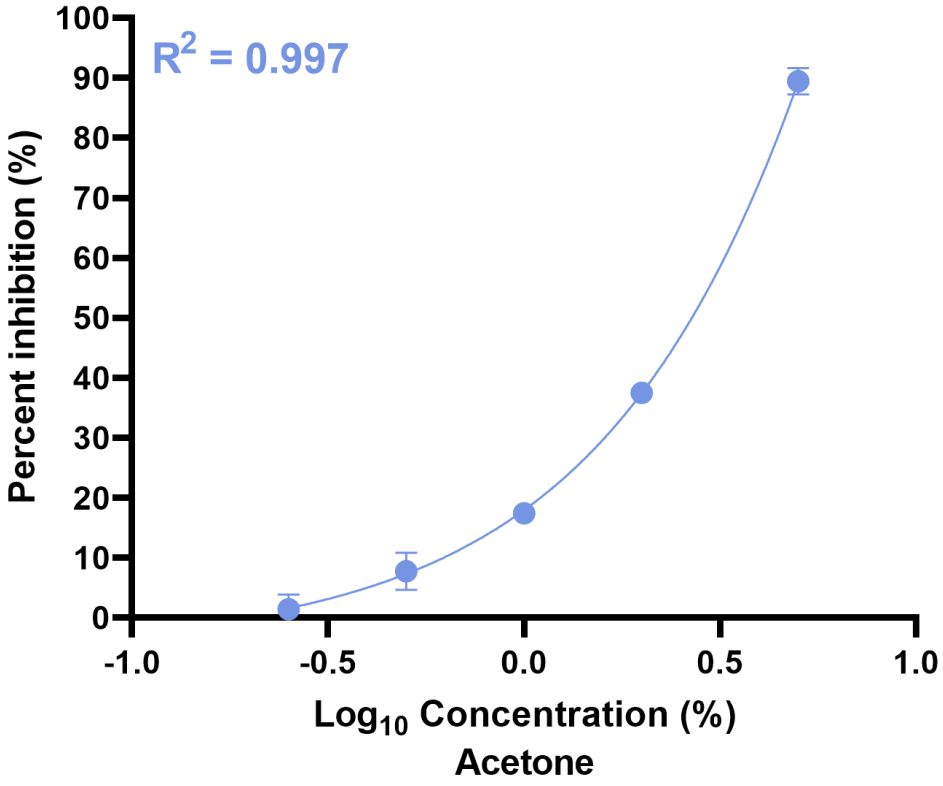

Figure B.1: Mycelial growth inhibition of *Phytophthora agathidicida* isolate ICMP 18970 with increasing concentrations of acetone
Error bars depict the standard error based on three replicates.

Table B.1: Full gas chromatography-mass spectrometry (GCMS) results for *Thymus vulargis* essential oil
No best match is provided for compounds not in the GC-MS database. For compounds that have a best match of 0-00-0, the structure predicted via GC-MS has no assigned CAS number.

Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
1	8.045	921	454180	0.07%	96	2867-05-2
2	8.363	925	3739459	0.57%	97	80-56-8
3	9.306	938	319283	0.05%	95	79-92-5
4	11.436	968	13529546	2.07%	97	18172-67-3
5	13.062	991	2606394	0.40%	96	123-35-3
6	13.752	1001	100276	0.02%	93	2867-05-2
7	14.136	1006	250854	0.04%	97	13466-78-9
8	14.698	1014	266124	0.04%	95	29050-33-7
9	15.388	1023	148384417	22.66%	97	99-87-6
10	15.552	1026	3398543	0.52%	93	555-10-2
11	15.762	1029	245311	0.04%	89	470-82-6
12	17.969	1059	198239166	30.28%	97	99-85-4
13	18.549	1067	154325	0.02%	89	17699-16-0
14	19.68	1083	108890	0.02%	88	99-85-4
15	19.855	1085	267648	0.04%	95	554-61-0
16	19.992	1087	528230	0.08%	94	2039-90-9
17	20.647	1096	171046	0.03%	88	546-79-2
18	21.649	1112	114248	0.02%	85	30434-65-2
19	23.1	1135	65738	0.01%		
20	23.317	1138	339998	0.05%	88	460-01-5
21	23.79	1145	216940	0.03%	81	0-00-0
22	23.952	1148	193533	0.03%	89	43219-68-7
23	24.191	1152	313093	0.05%	88	491-07-6
24	24.809	1162	269815	0.04%	86	74063-72-2
25	25.069	1166	3718916	0.57%	88	0-00-0
26	25.136	1167	5669113	0.87%	88	1195-92-2
27	25.425	1171	177721	0.03%	87	76109-40-5
28	25.605	1174	1146892	0.18%	95	562-74-3
29	26.378	1186	510962	0.08%	95	1197-01-9
30	26.641	1190	641271	0.10%	93	10482-56-1
31	28.189	1217	171357	0.03%		
32	28.555	1223	176544	0.03%	95	7492-41-3
33	29.509	1240	113127	0.02%		
34	30.343	1254	104915	0.02%		
35	31.842	1280	85592	0.01%	81	0-00-0
36	32.054	1284	170730	0.03%	90	9/04/4821
37	32.623	1294	1782747	0.27%	97	89-83-8
38	32.767	1297	864251	0.13%	96	89-83-8
39	33.125	1303	257742406	39.37%	97	89-83-8
40	33.412	1309	2528256	0.39%	96	499-75-2
41	33.723	1315	193976	0.03%		
42	34.074	1321	349168	0.05%	93	104-45-0

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
43	34.326	1326	63407	0.01%		
44	34.606	1331	276177	0.04%	93	22539-72-6
45	34.801	1335	126652	0.02%		
46	35.098	1341	71902	0.01%	85	491-09-8
47	36.663	1370	160676	0.02%	94	88-60-8
48	37.622	1389	150716	0.02%	89	1879-09-0
49	39.494	1426	69379	0.01%		
50	39.785	1432	108515	0.02%	84	40625-96-5
51	44.208	1523	43734	0.01%	83	484-34-4
52	46.665	1576	36856	0.01%	84	1139-30-6
53	47.308	1589	80814	0.01%	86	465-28-1
54	47.664	1597	252580	0.04%	96	84-66-2
55	47.901	1602	92806	0.01%		
56	54.842	1763	159987	0.02%		
57	58.233	1846	147264	0.02%	92	502-69-2
58	60.099	1893	122949	0.02%		
59	60.345	1899	378986	0.06%		
60	61.16	1920	144761	0.02%	80	0-00-0
61	61.458	1928	86954	0.01%		
62	63.209	1974	90184	0.01%		
63	63.357	1978	240276	0.04%	81	60713-96-4
64	63.564	1984	79305	0.01%		
65	63.997	1995	65377	0.01%		
66	64.26	2002	128885	0.02%		
67	65.271	2028	56092	0.01%		
68	65.84	2043	160471	0.02%		
69	67.002	2074	82055	0.01%	83	27417-37-4
70	68.311	2108	512194	0.08%	84	0-00-0
71	69.643	2143	27826	0.00%		
72	70.158	2157	24118	0.00%		
73	70.452	2164	95452	0.01%		
74	70.912	2176	40674	0.01%		
75	71.155	2183	50834	0.01%		
76	71.271	2186	62744	0.01%		

Table B.2: Full gas chromatography-mass spectrometry (GCMS) results for *Melaleuca alternifolia* essential oil
No best match is provided for compounds not in the GC-MS database. For compounds that have a best match of 0-00-0, the structure predicted via GC-MS has no assigned CAS number.

Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
1	8.055	921	3904455	0.62%	96	2867-05-2
2	8.386	925	14466763	2.29%	97	80-56-8
3	9.345	939	58541	0.01%	93	5794-04-7
4	11.448	968	7287595	1.16%	96	127-91-3
5	13.085	991	5073253	0.80%	95	123-35-3
6	13.749	1001	2239007	0.36%	96	99-83-2
7	14.717	1014	52385665	8.31%	97	99-86-5
8	15.324	1022	10007971	1.59%	97	25155-15-1
9	15.588	1026	11875858	1.88%	91	1461-27-4
10	15.746	1028	86499900	13.72%	96	0-00-0
11	17.932	1059	102366671	16.23%	97	99-85-4
12	18.515	1067	522508	0.08%	96	17699-16-0
13	19.86	1085	18677278	2.96%	97	586-62-9
14	20.075	1088	155698	0.02%	87	2039-90-9
15	20.596	1096	1750018	0.28%	96	17699-16-0
16	21.073	1103	250488	0.04%	95	78-70-6
17	22.11	1119	1605194	0.25%	94	29803-81-4
18	23.142	1135	104156	0.02%	93	586-82-3
19	23.368	1139	1045898	0.17%	95	35376-39-7
20	25.193	1168	282931	0.04%	90	10482-56-1
21	25.813	1177	211676794	33.57%	97	562-74-3
22	26.39	1187	529229	0.08%	83	1197-01-9
23	26.579	1190	21507244	3.41%	98	98-55-5
24	26.824	1193	856347	0.14%	91	16721-39-4
25	27.66	1207	525527	0.08%	92	16721-38-3
26	27.83	1210	72103	0.01%		
27	30.137	1251	143798	0.02%		
28	31.218	1270	247111	0.04%	92	0-00-0
29	32.301	1289	135076	0.02%	86	0-00-0
30	34.132	1322	187464	0.03%		
31	34.661	1332	66931	0.01%	89	3242-08-8
32	35.343	1345	396425	0.06%	96	17699-14-8
33	36.491	1367	334697	0.05%	94	95910-36-4
34	36.637	1370	702640	0.11%	95	3856-25-5
35	36.808	1373	411704	0.07%	91	27862-07-3
36	36.927	1375	201687	0.03%	88	27862-07-3
37	37.453	1385	53385	0.01%	87	23986-74-5
38	37.586	1388	162950	0.03%	91	515-13-9
39	37.663	1389	184464	0.03%	87	489-39-4
40	38.338	1402	2242523	0.36%	96	489-40-7
41	38.495	1405	72274	0.01%	86	489-40-7

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
42	38.618	1408	122779	0.02%	87	0-00-0
43	38.787	1411	3444800	0.55%	96	87-44-5
44	38.975	1415	69652	0.01%		
45	39.253	1421	389505	0.06%	91	25246-27-9
46	39.396	1424	112213	0.02%	88	17334-55-3
47	39.52	1426	391517	0.06%	91	6813-21-4
48	39.771	1431	7612066	1.21%	96	489-39-4
49	40.016	1436	1108868	0.18%	84	3691-11-0
50	40.394	1444	1547916	0.25%	87	0-00-0
51	40.495	1446	837188	0.13%	91	6753-98-6
52	40.861	1453	3386489	0.54%	97	25246-27-9
53	41.02	1457	280889	0.04%	91	489-40-7
54	41.486	1466	313991	0.05%	92	22567-17-5
55	41.612	1469	2784231	0.44%	92	483-76-1
56	41.788	1472	230161	0.04%	91	23515-88-0
57	42.124	1479	475698	0.08%	95	17066-67-0
58	42.234	1481	616381	0.10%	92	0-00-0
59	42.401	1485	1152826	0.18%	92	54274-73-6
60	42.487	1486	798046	0.13%	84	473-14-3
61	42.67	1490	12103904	1.92%	91	100762-46-7
62	42.986	1497	1429311	0.23%	96	10208-80-7
63	43.285	1503	243899	0.04%	90	489-40-7
64	43.58	1509	287747	0.05%	93	30021-74-0
65	44.105	1520	10904138	1.73%	96	483-76-1
66	44.445	1528	1577164	0.25%	96	16728-99-7
67	45.273	1546	135184	0.02%	85	552-02-3
68	45.653	1554	920221	0.15%	92	552-02-3
69	45.85	1558	138695	0.02%	84	523-47-7
70	45.971	1561	867560	0.14%	87	5986-49-2
71	46.514	1572	693709	0.11%	93	6750-60-3
72	46.783	1578	3583265	0.57%	92	552-02-3
73	46.985	1582	330695	0.05%	88	0-00-0
74	47.123	1585	1509920	0.24%	94	552-02-3
75	47.261	1588	1461186	0.23%	82	0-00-0
76	47.635	1596	1513363	0.24%	87	63891-61-2
77	48.574	1618	1332514	0.21%	89	0-00-0
78	48.85	1624	2355134	0.37%	84	21996-77-0
79	49.332	1635	347445	0.06%	84	6750-60-3
80	49.474	1638	1378685	0.22%	87	0-00-0
81	49.717	1644	482662	0.08%	93	133645-25-7
82	49.87	1647	43010	0.01%		

Table B.3: Full gas chromatography-mass spectrometry (GCMS) results for *Pelargonium graveolens* essential oil.

No best match is provided for compounds not in the GC-MS database. For compounds that have a best match of 0-00-0, the structure predicted via GC-MS has no assigned CAS number.

Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
1	3.873	819	74959	0.01%	88	0-00-0
2	4.634	842	117676	0.02%	95	589-35-5
3	5.797	876	39740	0.01%	87	68752-16-9
4	8.369	925	2374584	0.46%	97	80-56-8
5	11.354	967	350835	0.07%	93	7392-19-0
6	12.805	987	171975	0.03%	91	514-95-4
7	13.079	991	601791	0.12%	88	106-25-2
8	13.748	1001	155992	0.03%	94	0-00-0
9	14.254	1008	103932	0.02%	93	13679-86-2
10	15.316	1022	397344	0.08%	97	527-84-4
11	15.556	1026	860457	0.17%	97	5989-27-5
12	15.814	1029	158462	0.03%	85	343855-44-7
13	15.932	1031	127793	0.02%	87	0-00-0
14	16.685	1041	218006	0.04%	93	6874-10-8
15	16.805	1043	59349	0.01%		
16	17.37	1051	229694	0.04%	96	3779-61-1
17	18.641	1068	69782	0.01%	83	586-82-3
18	18.884	1072	1372033	0.27%	98	5989-33-3
19	19.853	1085	84831	0.02%	94	586-62-9
20	20.017	1088	705424	0.14%	96	0-00-0
21	20.996	1101	22486367	4.37%	98	78-70-6
22	21.555	1110	5994817	1.16%	97	0-00-0
23	22.585	1126	2364092	0.46%	96	0-00-0
24	23.248	1137	81554	0.02%	87	106-22-9
25	23.608	1143	430282	0.08%	95	50373-36-9
26	23.944	1148	90627	0.02%	87	590-71-6
27	24.093	1150	13933360	2.71%	98	491-07-6
28	24.434	1156	336667	0.07%	96	2385-77-5
29	24.736	1160	23724128	4.61%	98	1196-31-2
30	25.625	1174	285968	0.06%	93	562-74-3
31	25.829	1178	69009	0.01%		
32	25.991	1180	805303	0.16%	97	0-00-0
33	26.365	1186	101825	0.02%		
34	26.528	1189	1776600	0.34%	97	98-55-5
35	27.633	1207	194301	0.04%		
36	27.837	1210	138287	0.03%		
37	28.612	1224	246321	0.05%	92	6812-78-8
38	29.005	1231	781757	0.15%	94	106-25-2
39	29.323	1236	167117415	32.45%	98	106-22-9
40	29.671	1242	5331883	1.04%	97	106-26-3
41	30.18	1251	144523	0.03%		

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
42	30.279	1253	215023	0.04%	92	89-81-6
43	30.5	1257	165678	0.03%	90	16052-42-9
44	30.731	1261	71666723	13.91%	96	106-24-1
45	31.293	1271	55457	0.01%		
46	31.416	1273	3448277	0.67%	98	141-27-5
47	31.723	1278	39490749	7.67%	97	105-85-1
48	31.988	1283	462575	0.09%	91	106-25-2
49	33.171	1304	14388573	2.79%	94	105-86-2
50	34.398	1327	163624	0.03%		
51	35.322	1345	1212858	0.24%	94	17699-14-8
52	35.786	1354	69134	0.01%	91	122-70-3
53	35.934	1357	1866990	0.36%	97	150-84-5
54	36.105	1360	87707	0.02%	83	22469-52-9
55	36.393	1365	314247	0.06%	94	14912-44-8
56	36.623	1370	3102100	0.60%	96	3856-25-5
57	36.795	1373	136081	0.03%		
58	37.041	1378	6597813	1.28%	98	5208-59-3
59	37.263	1382	233337	0.05%	87	17699-14-8
60	37.537	1387	2486296	0.48%	97	141-12-8
61	37.96	1395	225966	0.04%	95	103-52-6
62	38.178	1399	88434	0.02%	83	489-39-4
63	38.322	1402	375846	0.07%	94	489-40-7
64	38.775	1411	6581210	1.28%	96	87-44-5
65	39.315	1422	635364	0.12%	95	13744-15-5
66	39.513	1426	85017	0.02%		
67	39.757	1431	438941	0.09%	94	489-39-4
68	39.86	1433	191226	0.04%		
69	40.092	1438	2076700	0.40%	92	0-00-0
70	40.322	1442	2130817	0.41%	90	25246-27-9
71	40.48	1446	3783013	0.73%	87	141-14-0
72	40.666	1449	104589	0.02%	82	22469-52-9
73	40.844	1453	889995	0.17%	96	25246-27-9
74	41.127	1459	200724	0.04%	94	23986-74-5
75	41.395	1464	476156	0.09%	82	0-00-0
76	41.597	1468	806572	0.16%	90	483-76-1
77	41.774	1472	770302	0.15%	94	30021-74-0
78	41.887	1474	3874721	0.75%	96	23986-74-5
79	42.038	1477	5399946	1.05%	94	105-90-8
80	42.23	1481	9796	0.00%		
81	42.473	1486	135815	0.03%		
82	42.657	1490	3794952	0.74%	90	15423-57-1
83	42.968	1496	1700601	0.33%	95	31983-22-9
84	43.266	1502	303205	0.06%	90	483-76-1
85	43.557	1509	2058174	0.40%	95	30021-74-0
86	43.904	1516	425965	0.08%	93	106-29-6

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
87	44.077	1520	7302116	1.42%	95	483-76-1
88	44.44	1528	530643	0.10%	89	16728-99-7
89	44.552	1530	3221927	0.63%	96	141-16-2
90	44.696	1533	633291	0.12%	89	489-40-7
91	44.892	1537	2185146	0.42%		
92	45.175	1543	128271	0.02%		
93	45.293	1546	240263	0.05%	82	21284-22-0
94	45.451	1549	158299	0.03%	91	15423-57-1
95	45.76	1556	229021	0.04%	82	83489-22-9
96	45.944	1560	275903	0.05%		
97	46.087	1563	4549198	0.88%	96	106-29-6
98	46.496	1572	1496427	0.29%	95	6750-60-3
99	46.662	1575	986072	0.19%	93	1139-30-6
100	46.761	1578	520507	0.10%	86	489-41-8
101	46.954	1582	120707	0.02%		
102	47.126	1585	4736598	0.92%	95	55719-85-2
103	47.469	1593	87434	0.02%		
104	47.631	1596	568740	0.11%	85	63891-61-2
105	47.872	1602	531143	0.10%	82	473-15-4
106	47.993	1604	492276	0.10%	92	68705-63-5
107	48.37	1613	23030505	4.47%	97	0-00-0
108	48.555	1617	279783	0.05%		
109	48.835	1624	779690	0.15%	82	21996-77-0
110	48.945	1626	1149714	0.22%	90	141-14-0
111	49.157	1631	976162	0.19%	91	1460-73-7
112	49.319	1635	395768	0.08%	85	6750-60-3
113	49.552	1640	3310407	0.64%		
114	49.739	1644	1417628	0.28%	97	473-15-4
115	49.901	1648	1507071	0.29%	91	489-86-1
116	50.042	1651	881677	0.17%	88	481-34-5
117	50.285	1656	109672	0.02%		
118	50.41	1659	804040	0.16%	95	109-20-6
119	50.769	1668	1563278	0.30%	94	24717-85-9
120	50.925	1671	43831	0.01%		
121	51.576	1686	539102	0.10%	90	141-14-0
122	51.758	1690	55410	0.01%	85	473-04-1
123	52.09	1698	48223	0.01%		
124	52.331	1703	5573683	1.08%	94	7785-33-3
125	52.719	1712	69855	0.01%	82	77171-55-2
126	53.03	1720	983708	0.19%	92	10032-02-7
127	54.564	1756	364914	0.07%	94	10032-02-7
128	57.131	1819	386704	0.08%	90	141-14-0
129	58.231	1846	101880	0.02%	90	502-69-2
130	58.531	1854	321973	0.06%	89	109-20-6
131	59.114	1868	270320	0.05%	95	84-69-5

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
133	60.996	1916	105671	0.02%	90	0-00-0
134	62.34	1951	118455	0.02%	87	109-20-6
135	64.56	2010	51522	0.01%	83	0-00-0
136	65.888	2045	136294	0.03%	85	0-00-0
137	71.514	2192	508617	0.10%		
138	71.624	2195	51693	0.01%		
139	71.728	2198	63921	0.01%		
140	71.944	2204	30729	0.01%		
141	72.075	2207	354441	0.07%	81	0-00-0
142	72.206	2210	87277	0.02%		
143	72.283	2212	125331	0.02%		
144	72.37	2215	108671	0.02%	81	7683-64-9
145	72.764	2225	203565	0.04%		
146	72.957	2230	111889	0.02%		
147	73.092	2234	16894	0.00%		
148	73.346	2240	47945	0.01%		
149	73.496	2244	50925	0.01%		
150	73.644	2248	91343	0.02%		
151	74.475	2270	23306	0.00%		
152	75.86	2306	40083	0.01%		
153	76.056	2311	282242	0.05%	88	0-00-0

Table B.4: Full gas chromatography-mass spectrometry (GCMS) results for mānuka (*Leptospermum scoparium*) essential oil

No best match is provided for compounds not in the GC-MS database. For compounds that have a best match of 0-00-0, the structure predicted via GC-MS has no assigned CAS number.

Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
1	3.2	799	48521	0.01%	95	565-80-0
2	3.269	801	58523	0.01%	93	589-43-5
3	8.065	921	111831	0.02%	95	2/05/2867
4	8.372	925	7052490	1.09%	97	80-56-8
5	11.46	968	1126164	0.17%	97	18172-67-3
6	13.082	991	1225096	0.19%	96	123-35-3
7	14.738	1014	93776	0.01%	85	554-61-0
8	15.315	1022	992194	0.15%	97	25155-15-1
9	15.575	1026	490953	0.08%	96	5989-27-5
10	15.714	1028	836900	0.13%	96	470-82-6
11	17.404	1051	136331	0.02%	94	3338-55-4
12	17.854	1058	632744	0.10%	96	99-85-4
13	19.867	1085	203596	0.03%	95	586-62-9
14	21.06	1102	698955	0.11%	84	78-70-6
15	21.46	1109	784142	0.12%	98	659-70-1
16	21.648	1112	397839	0.06%	95	25415-62-7
17	21.993	1117	76915	0.01%	95	84254-81-9
18	22.197	1120	1173692	0.18%	96	54410-94-5
19	24.357	1154	117156	0.02%	90	0-00-0
20	24.84	1162	195033	0.03%	85	7333-23-5
21	25.036	1165	480543	0.07%	89	5/07/7307
22	25.638	1175	208680	0.03%	95	562-74-3
23	26.581	1190	257912	0.04%	96	10482-56-1
24	27.054	1197	1040579	0.16%	94	41519-18-0
25	27.609	1206	280919	0.04%	95	0-00-0
26	30.112	1250	254644	0.04%	84	0-00-0
27	31.057	1267	154881	0.02%	81	0-00-0
28	31.769	1279	894411	0.14%	86	69668-83-3
29	35.35	1345	22864292	3.52%	97	17699-14-8
30	36.402	1365	1876749	0.29%	95	14912-44-8
31	36.658	1370	29993282	4.62%	96	3856-25-5
32	36.807	1373	80098	0.01%	87	6831-16-9
33	37.055	1378	267080	0.04%	93	5208-59-3
34	37.215	1381	190091	0.03%	92	515-13-9
35	37.441	1385	656949	0.10%	90	13744-15-5
36	37.575	1388	7305898	1.13%	96	515-13-9
37	37.921	1394	1238402	0.19%	95	10361-39-4
38	38.179	1399	122301	0.02%		
39	38.337	1402	7061296	1.09%	96	489-40-7
40	38.479	1405	131282	0.02%	91	489-40-7
41	38.619	1408	43831	0.01%	83	18431-82-8

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
42	38.801	1412	22498782	3.47%	96	87-44-5
43	39.332	1422	1714835	0.26%	95	13744-15-5
44	39.508	1426	545003	0.08%	81	56633-28-4
45	39.769	1431	11377018	1.75%	96	489-39-4
46	40.01	1436	1253541	0.19%	89	94-46-2
47	40.118	1438	1182981	0.18%	88	6831-16-9
48	40.418	1445	33231423	5.12%	90	0-00-0
49	40.697	1450	39549	0.01%		
50	40.86	1453	4357151	0.67%	96	25246-27-9
51	41.002	1456	445296	0.07%	86	475-20-7
52	41.325	1463	1002376	0.15%	93	0-00-0
53	41.64	1469	29242955	4.51%	92	483-76-1
54	41.817	1473	6314485	0.97%	92	30021-74-0
55	41.985	1476	1955395	0.30%	94	483-75-0
56	42.176	1480	49603167	7.64%	96	17066-67-0
57	42.417	1485	5636238	0.87%	93	54274-73-6
58	42.643	1490	50248451	7.74%	96	473-13-2
59	42.994	1497	10070042	1.55%	96	10208-80-7
60	43.287	1503	987503	0.15%	92	483-76-1
61	43.624	1510	13170569	2.03%	92	502-61-4
62	44.173	1522	146437856	22.57%	94	483-77-2
63	44.511	1529	37830202	5.83%	96	16728-99-7
64	44.721	1534	1004518	0.15%	93	24406-05-1
65	44.859	1537	3457399	0.53%		
66	44.942	1538	5258051	0.81%	88	0-00-0
67	45.172	1543	3482747	0.54%		
68	45.318	1547	1026274	0.16%	82	639-99-6
69	45.477	1550	299922	0.05%	89	15423-57-1
70	45.658	1554	727491	0.11%	90	552-02-3
71	45.895	1559	544051	0.08%	89	0-00-0
72	46.057	1562	1735605	0.27%	No	
73	46.146	1564	1145654	0.18%	92	7212-44-4
74	46.358	1569	202252	0.03%		
75	46.514	1572	4516868	0.70%	94	6750-60-3
76	46.683	1576	5143396	0.79%	92	1139-30-6
77	46.782	1578	2700661	0.42%	91	489-41-8
78	46.976	1582	2197592	0.34%	91	0-00-0
79	47.119	1585	1203302	0.19%	92	552-02-3
80	47.249	1588	1025520	0.16%	80	13822-35-0
81	47.442	1592	712013	0.11%	No	
82	47.633	1596	3465956	0.53%	88	577-27-5
83	47.897	1602	1093081	0.17%	85	6975-94-6
84	48.133	1608	2445501	0.38%	84	25269-17-4
85	48.245	1610	409921	0.06%	No	
86	48.401	1614	697830	0.11%	81	0-00-0

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
87	48.604	1618	8681897	1.34%		
88	48.859	1624	9358048	1.44%	85	21996-77-0
89	48.977	1627	17924218	2.76%		
90	49.191	1632	824139	0.13%	82	0-00-0
91	49.357	1635	2180371	0.34%	81	0-00-0
92	49.484	1638	11340148	1.75%	87	0-00-0
93	49.769	1645	7736494	1.19%	97	473-15-4
94	49.922	1648	11395129	1.76%	95	473-16-5
95	50.271	1656	1427213	0.22%	No	
96	50.423	1660	754987	0.12%	No	
97	50.681	1665	734402	0.11%	88	0-00-0
98	50.907	1671	1658720	0.26%	93	483-78-3
99	51.08	1675	3311758	0.51%	86	0-00-0
100	51.448	1683	259616	0.04%		
101	51.63	1687	73699	0.01%		
102	51.762	1690	166125	0.03%		
103	52.089	1698	157256	0.02%		
104	52.235	1701	68236	0.01%		
105	52.415	1705	382698	0.06%	81	0-00-0
106	52.816	1715	310312	0.05%		
107	52.918	1717	278911	0.04%		
108	53.086	1721	1211074	0.19%	82	488-10-8
109	53.239	1725	150176	0.02%	90	0-00-0
110	53.625	1734	90441	0.01%		
111	53.895	1740	166396	0.03%		
112	54.081	1745	105310	0.02%		
113	54.69	1759	66454	0.01%		
114	54.836	1763	218564	0.03%		
115	55.009	1767	227660	0.04%	81	133610-00-1
116	55.737	1784	588073	0.09%	80	24268-34-6
117	56.309	1798	158153	0.02%		
118	56.44	1801	35404	0.01%		
119	56.565	1804	465100	0.07%	82	1012-72-2
120	57.318	1823	144726	0.02%		
121	57.591	1830	199796	0.03%		
122	58.243	1846	54978	0.01%	86	502-69-2
123	58.66	1857	61468	0.01%		
124	70.111	2155	53397	0.01%		
125	70.254	2159	23430	0.00%		
126	70.449	2164	59772	0.01%		
127	71.724	2198	25269	0.00%	82	629-92-5
128	72.316	2213	48550	0.01%		
129	72.51	2218	74951	0.01%		
130	72.824	2227	104033	0.02%		
131	72.961	2230	49950	0.01%		

Table B.5: Full gas chromatography-mass spectrometry (GCMS) results for kānuka (*Kunzea ericoides*) essential oil

No best match is provided for compounds not in the GC-MS database. For compounds that have a best match of 0-00-0, the structure predicted via GC-MS has no assigned CAS number.

Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
1	3.172	798	1525295	0.27%	98	565-80-0
2	3.395	804	32835	0.01%	90	38667-10-6
3	3.622	811	30448	0.01%	94	3212-68-8
4	4.117	826	297674	0.05%	95	0-00-0
5	4.988	852	753743	0.13%	98	503-74-2
6	5.38	864	87560	0.02%	93	75-65-0
7	5.718	874	907837	0.16%	98	123-92-2
8	8.06	921	3325269	0.58%	96	2867-05-2
9	8.519	927	303421495	53.38%	97	80-56-8
10	9.32	938	644688	0.11%	97	79-92-5
11	9.825	946	159078	0.03%	94	0-00-0
12	11.449	968	2986522	0.53%	97	18172-67-3
13	13.096	991	350020	0.06%	86	51152-12-6
14	14.262	1008	111532	0.02%	93	13679-86-2
15	14.759	1015	536772	0.09%	86	17334-05-3
16	14.971	1018	155066	0.03%	91	106-27-4
17	15.306	1022	38813776	6.83%	97	25155-15-1
18	15.568	1026	5514216	0.97%	95	5989-27-5
19	15.687	1027	42324954	7.45%	96	0-00-0
20	17.404	1051	72137	0.01%	92	3338-55-4
21	17.841	1057	1462767	0.26%	97	99-85-4
22	18.141	1062	418161	0.07%	96	60415-61-4
23	18.741	1070	1334105	0.23%	80	5333-84-6
24	18.902	1072	818261	0.14%	94	5989-33-3
25	19.846	1085	670957	0.12%	96	586-62-9
26	19.997	1087	1509397	0.27%	85	1587-04-8
27	20.165	1090	136411	0.02%	86	33171-49-2
28	20.334	1092	1310340	0.23%	95	1686-14-2
29	20.846	1099	146551	0.03%	80	82061-20-9
30	20.98	1101	13146564	2.31%	97	78-70-6
31	21.465	1109	892926	0.16%	97	659-70-1
32	21.758	1113	151556	0.03%	91	546-80-5
33	22.226	1121	166622	0.03%	93	54410-94-5
34	22.393	1123	560390	0.10%	94	4501-58-0
35	23.027	1133	2698323	0.47%	96	547-61-5
36	23.65	1143	663430	0.12%	88	18881-04-4
37	23.76	1145	482310	0.08%	85	0-00-0
38	24.479	1156	239653	0.04%	94	547-60-4
39	24.64	1159	84868	0.01%	87	30460-92-5
40	24.875	1163	426621	0.08%	97	507-70-0
41	25.094	1166	389574	0.07%	90	10482-56-1

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
42	25.311	1169	114598	0.02%	88	15358-88-0
43	25.586	1174	3074105	0.54%	96	562-74-3
44	26.308	1185	1126742	0.20%	95	1197-01-9
45	26.513	1188	9268438	1.63%	98	98-55-5
46	26.688	1191	321524	0.06%	90	564-94-3
47	26.894	1194	180244	0.03%	95	515-00-4
48	27.552	1205	685850	0.12%	96	80-57-9
49	28.328	1219	972811	0.17%	98	1197-06-4
50	28.57	1223	78281	0.01%		
51	29.079	1232	94287	0.02%	90	1197-06-4
52	29.709	1243	152119	0.03%	93	99-49-0
53	30.108	1250	243100	0.04%		
54	30.312	1254	98709	0.02%	87	2035-99-6
55	30.583	1258	1058702	0.19%	98	103-45-7
56	31.564	1276	137217	0.02%	83	817-88-9
57	31.939	1282	101191	0.02%		
58	32.275	1288	64046	0.01%		
59	32.419	1291	145788	0.03%		
60	32.821	1298	600052	0.11%	85	97631-68-0
61	33.225	1305	76169	0.01%		
62	33.411	1309	367929	0.06%	80	24545-81-1
63	33.854	1317	218113	0.04%	92	1845-30-3
64	35.326	1345	1778196	0.31%	96	17699-14-8
65	36.398	1365	245217	0.04%	85	14912-44-8
66	36.471	1367	254929	0.04%	87	0-00-0
67	36.624	1370	4901285	0.86%	95	3856-25-5
68	36.795	1373	578531	0.10%	92	6831-16-9
69	36.95	1376	56909	0.01%		
70	37.415	1385	48962	0.01%		
71	37.554	1387	989072	0.17%	88	141-12-8
72	37.924	1394	432043	0.08%	92	103-38-8
73	38.325	1402	2460328	0.43%	96	489-40-7
74	38.772	1411	1306969	0.23%	96	87-44-5
75	39.24	1421	176350	0.03%	86	473-13-2
76	39.383	1424	379898	0.07%	92	17334-55-3
77	39.505	1426	226261	0.04%	83	6813-21-4
78	39.756	1431	3578726	0.63%	94	489-39-4
79	39.848	1433	1712445	0.30%	89	489-39-4
80	40.004	1436	272879	0.05%		
81	40.1	1438	640535	0.11%	91	27862-07-3
82	40.29	1442	288225	0.05%	88	103-48-0
83	40.486	1446	829210	0.15%	94	6753-98-6
84	40.847	1453	4391889	0.77%	97	25246-27-9
85	41.005	1456	235773	0.04%	90	0-00-0
86	41.315	1463	45298	0.01%		

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
87	41.474	1466	432440	0.08%	92	22567-17-5
88	41.6	1468	274093	0.05%		
89	41.68	1470	291942	0.05%	82	0-00-0
90	41.776	1472	761362	0.13%	93	30021-74-0
91	41.94	1475	119800	0.02%	92	23515-88-0
92	42.108	1479	1887917	0.33%	96	17066-67-0
93	42.227	1481	2153484	0.38%	87	0-00-0
94	42.616	1489	9427662	1.66%	95	21747-46-6
95	42.972	1496	1213844	0.21%	96	10208-80-7
96	43.089	1499	467019	0.08%	83	0-00-0
97	43.561	1509	1322205	0.23%	96	30021-74-0
98	43.74	1513	93295	0.02%		
99	44.053	1519	25628185	4.51%	94	483-77-2
100	44.435	1528	1201267	0.21%	96	16728-99-7
101	44.687	1533	265257	0.05%	86	483-75-0
102	44.837	1536	458004	0.08%		
103	44.915	1538	671285	0.12%	81	0-00-0
104	45.318	1547	2144182	0.38%	82	0-00-0
105	45.641	1553	265418	0.05%	91	552-02-3
106	45.968	1561	2786212	0.49%	95	5986-49-2
107	46.142	1564	3737541	0.66%	96	40716-66-3
108	46.36	1569	58633	0.01%		
109	46.505	1572	5007867	0.88%	95	6750-60-3
110	46.669	1576	1156953	0.20%	92	1139-30-6
111	46.789	1578	1589856	0.28%	91	489-41-8
112	47.133	1586	16906915	2.97%	95	552-02-3
113	47.252	1588	1534109	0.27%	81	941-37-7
114	47.467	1593	99194	0.02%		
115	47.631	1596	5747716	1.01%	92	577-27-5
116	47.893	1602	249681	0.04%	84	6975-94-6
117	48.022	1605	121064	0.02%		
118	48.57	1618	512875	0.09%	90	0-00-0
119	48.841	1624	643631	0.11%	84	21996-77-0
120	48.956	1626	1195725	0.21%		
121	49.324	1635	198688	0.03%		
122	49.46	1638	1057881	0.19%	85	19435-97-3
123	49.711	1643	203500	0.04%	90	19435-97-3
124	49.845	1646	87229	0.02%		
125	50.038	1651	250982	0.04%	83	481-34-5
126	50.27	1656	444133	0.08%		
127	50.425	1660	56715	0.01%		
128	50.673	1665	231742	0.04%	85	0-00-0
129	50.91	1671	239293	0.04%	94	483-78-3
130	51.328	1680	158362	0.03%		
131	53.091	1721	108930	0.02%		

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Compound #	R.T. (mins)	Measured RI	Peak area	Area %	Hit %	Best match (CAS No.)
132	54.699	1760	57272	0.01%		
133	55.737	1784	170819	0.03%		

Appendix C

Supplemental data for Chapter 4

Table C.1: PCR reaction mixture for using universal oomycete primers

Reagent	Volume (µL)
MyTaq™ Red Reaction Buffer	5
10 µM ITS-4 5'-TCCTCCGCTTATTGATATGC-3	0.5
10 µM ITS-6 5'-GAAGGTGAAGTCGTAACAAGG-3	0.5
MyTaq™ HS Red DNA Polymerase	0.2
Nuclease-free water	17.8
DNA	1

Table C.2: PCR reaction mixture for using *Phytophthora agathidicida* specific primers

Reagent	Volume (µL)
MyTaq™ Red Reaction Buffer	5
10 µM ITS F 5'- AACCAATAGTTGGGGGCGA-3	0.5
10 µM ITS R 5'- CTCGCCATGATAGAGCTCGTC-3	0.5
MyTaq™ HS Red DNA Polymerase	0.2
Nuclease-free water	17.8
DNA	1